REMARKS

Applicants have amended Figs. 2-5 in order to delete the asterisks therefrom. In light of the amendments to the drawings figures, it is respectfully submitted that the objection to Figs. 2-5 as set forth in Item 3 on pages 2 and 3 of the Office Action mailed April 2, 2008, is moot. Moreover, with the present submission of Replacement Sheets 2/7 through 5/7, it is respectfully submitted that the requirement for corrected drawing sheets has been satisfied.

Applicants have amended their specification in light of the issues raised by the Examiner in objecting to the disclosure, in Item 2 on page 2 of the Office Action mailed April 2, 2008. In light of these amendments to the specification, it is respectfully submitted that the objection to the disclosure in Item 2 on page 2 of the Office Action mailed April 2, 2008, is moot, and that the required correction has been made.

Applicants have amended their claims in order to further clarify the definition of various aspects of the present invention. Specifically, Applicants have amended each of claims 1-3 to recite a "cell-mediated immunological" diagnostic method. Applicants have further amended claim 1 to recite that in collecting blood of a subject animal, collected blood is thereby provided; to recite a step of adding an anti-interleukin 10 (IL-10) antibody "to the collected blood, while inducing cell-mediated immunological reaction against *Mycobacterium avium* subsp. *paratuberculosis* in the collected blood"; to recite that the measuring is after the adding; and to delete recitation of culturing in the last line thereof.

Independent claim 3 has been further amended to recite that in collecting the blood of a subject animal, collected blood is provided; to recite the step of adding an anti-interleukin 10 (IL-10) antibody to the collected blood, while inducing cell-

mediated immunological reaction against mycobacterium in the collected blood; to recite that the measuring is subsequent to the adding; and to delete recitation of culturing in the last line thereof.

Moreover, Applicants are adding new claims 4-6 to the application. Claim 4, dependent on claim 1, recites that the cell-mediated immunological reaction against *Mycobacterium avium* subsp. *paratuberculosis* in the collected blood is induced by adding *Mycobacterium avium* subsp. *paratuberculosis* antigen selected from the group of *Mycobacterium avium* subsp. *paratuberculosis* PPD, live *Mycobacterium avium* subsp. *paratuberculosis* and soluble antigen obtained by heat-killed *Mycobacterium avium* subsp. *paratuberculosis* to the collected blood. See, e.g., the sole full paragraph on page 15 of Applicants specification. Claims 5 and 6, dependent respectively on claims 1 and 3, recite that the subject animal is cattle.

Applicants respectfully traverse the rejection of their claims under the second paragraph of 35 USC 112, as being incomplete for omitting essential steps, particularly insofar as this rejection is applicable to the claims as presently amended.

Thus, note that the present claims recite a cell-mediated immunological diagnostic method including, inter alia, adding an IL-10 antibody to the collected blood, while inducing cell-mediated immunological reaction against specified matter in the collected blood, with, subsequently, measuring an amount of produced interferon-γ in the blood. It is respectfully submitted that the claimed method corresponds to the processing described, for example, in the paragraph bridging pages 9 and 10, and the first full paragraph on page 10, of Applicants' specification, thereby showing that the claims particularly as presently amended do not omit essential steps.

Reference by the Examiner to omitted steps in the first three lines on page 4 of the Office Action mailed April 2, 2008, is noted. It is respectfully submitted that, especially as presently amended, the claims do not omit essential steps for the cell-mediated immunological diagnostic method as presently recited in the claims.

Applicants respectfully traverse the rejection of their claims under the first paragraph of 35 USC 112, as not being supported by an enabling disclosure, especially insofar as this rejection is applicable to the claims as presently amended. Thus, the Examiner contends that while the specification is enabling for distinguishing between infected and uninfected cattle by utilizing *M. avium* subsp. *paratuberculosis* PPD, the specification does not reasonably provide enablement for use of other single antigens. This contention by the Examiner is respectfully traversed. Thus, on page 13 of Applicants' specification, various subject animals are disclosed; and it is also described that there is involvement of a *Mycobacterium avium* subsp. *paratuberculosis* antigen in human Crohn's disease. Various antigens that can be used are described, for example, in the sole full paragraph on page 15 of Applicants' specification, with preparation thereof also being described. See also new claim 4. Contrary to the contention by the Examiner in the first paragraph of Item 6, on page 4 of the Office Action mailed April 2, 2008, as Applicants disclose use of other antigens from *Mycobacterium avium* subsp. *paratuberculosis*, it is respectfully submitted that Applicants need not be limited to the specific antigen alleged by the Examiner in the first paragraph of Item 6 on page 4 of the Office Action mailed April 2, 2008.

Moreover, while Applicants have described various specific antigens which can be used, the Examiner has provided no evidence or reasoning that Applicants are in error that such other antigens can be used. Absent evidence or reasoning

submitted by the Examiner that other antigens within the scope of the present claims can be used, as stated by Applicants in their specification, the enablement rejection is improper. See In re Dinh-Nguyen, 181 USPQ 46 (CCPA 1974); In re Bowen, 181 USPQ 48 (CCPA 1974).

Furthermore, it is emphasized that Applicants provide guidance in connection with the presently claimed cell-mediated immunological diagnostic method, providing specific examples. It is respectfully submitted that one of ordinary skill in the art using a specific antigen within the scope of the present claims, could easily determine whether such antigen can be used with the claimed method, including measuring an amount of produced interferon-γ. In view thereof, it is respectfully submitted that any experimentation necessary for practicing the claimed cell-mediated immunological diagnostic method would not be undue. See In re Angstadt, 190 USPQ 214 (CCPA 1976).

In the first paragraph on page 5 of the Office Action mailed April 2, 2008, the Examiner refers to the Koets, et al. article, as teaching that substituting a single antigen for PPD from *M. avium* subsp. *paratuberculosis* does not result in the same reactivity utilizing samples from cattle infected with *M. avium* subsp. *paratuberculosis*; and that, thus, there is a lack of predictability in the art that merely substituting PPD from *M. avium* subsp. *paratuberculosis* with any single antigen from *M. avium* subsp. *paratuberculosis* would result in the ability to diagnose infection utilizing the present methodology.

Generally, a single antigen used in Koets, et al. is known to have antigenicity and immune stimulation weaker than those of complex antigens such as PPD.

However, as seen in the Examples in the present application, a single protein or single peptide contained in PPD is considered to have a high possibility to induce

production of IFNγ. However, no single antigen showing antigenicity stronger than that of complex antigen has been known.

Cell-mediated immunological diagnostic methods including a step of selecting an appropriate antigen is a technique widely used to diagnose other diseases than paratuberculosis, and belongs to a common technique in the field of the present invention. This method can be carried out easily by those skilled in the art, based on routine experimental work.

In other words, the feature of the present invention is to enhance sensitivity in the prior technique of cell-mediated immunological diagnostic methods, by adding anti-IL-10 neutralizing antibody, which is based on a universal theory common to single and complex antigens. It is respectfully submitted that one of ordinary skill in the art could determine antigens within the scope of the present claims, for performing the claimed cell-mediated immunological diagnostic method. In view thereof, it is respectfully submitted that the present disclosure is sufficiently enabling to satisfy the requirements of the first paragraph of 35 USC 112, for one of ordinary skill in the art, with respect to subject matter of the present claims.

The contention by the Examiner that the specification only utilizes PPD as set forth in the second full paragraph on page 5 of the Office Action mailed April 2, 2008, is respectfully traversed. As described in the sole full paragraph on page 15 of Applicants' specification, other *Mycobacterium avium* subsp. *paratuberculosis* antigens can be used. See also claim 4.

The contention by the Examiner in the third full paragraph on page 5 of the Office Action mailed April 2, 2008, is respectfully traversed. It is respectfully submitted that in view of Applicants' disclosure as a whole, to one of ordinary skill in the art, and noting that cell-mediated immunological diagnostic methods including a

step of selecting appropriate antigens is a technique widely used to diagnose other diseases than paratuberculosis, and belongs to a common technique in the field of the present invention, it is respectfully submitted that choice of specific antigens within the present claims can be determined by one of ordinary skill in the art without undue experimentation. It is emphasized that the enablement requirement of 35 USC 112 does not prohibit all experimentation, only undue experimentation. See In re Angstadt, supra.

Applicants respectfully traverse the rejection of claim 3 under the first paragraph of 35 USC 112, as set forth in Item 7 on pages 5-7 of the Office Action mailed April 2, 2008, particularly insofar as this rejection is applicable to claim 3 as presently amended. Thus, claim 3 has been amended to recite a cell-mediated immunological diagnostic method, including the step, inter alia, of adding an anti-interleukin 10 (IL-10) antibody to the collected blood, while inducing cell-mediated immunological reaction against mycobacterium in the collected blood. As shown in the abstract of the article by Redpath, et al., "Hijacking an exploitation of IL-10 in intracellular pathogens", in Trends in Microbiology, Vol. 9, Issue 2 (1 February 2001), pages 86-92, a copy of which is enclosed herewith, it was known as of the date of the above-identified application that by allowing host macrophages to release IL-10, intracellular pathogens containing *Mycobacterium* shows strong immunosuppression.

Attention is also directed to the following articles enclosed herewith, each having a date prior to that of the above-identified application:

1. D. R. Roach, et al., "Endogenous Inhibition of Antimycobacterial Immunity by IL-10 Varies between Mycobacterial Species", Scand. J. Immunol., Vol. 54 (2001), pp. 163-170;

2. M. Jacobs, et al., "Enhanced immune response in Mycobacterium bovis bacille calmette guerin (BCG)-infected IL-10-deficient mice", Clin Chem Lab Med., Vol. 40, No. 9 (2002 Sept.), pp. 893-902;
3. J.-S. Lee, "Profiles of IFN-γ and its regulatory cytokines (IL-12, IL-18 and IL-10) in peripheral blood mononuclear cells from patients with multidrug-resistant tuberculosis", Clin Exp Immunnol, Vol. 128 (2002), pp. 516-524;
4. H. F. Geerdes-Fenge, et al., "Evaluation of the effect of interleukin-10 on the multiplication of Mycobacterium avium complex in human macrophages and in C57BL/6 mice", Clin microbiol Infect., Vol. 5, No. 9 (1999 Sept.), pp. 560-566;
5. J. Turner, et al., "In Vivo IL-10 Production Reactivates Chronic Pulmonary Tuberculosis in C57BL/6 Mice", The Journal of Immunology, Vol. 169 (2002), pp. 6343-6351; and
6. A. Fietta, et al., "Virulence of Mycobacterium tuberculosis affects interleukin-8, monocyte chemoattractant protein-1 and interleukin-10 production by human mononuclear phagocytes", Int J. Tissue React., Vol. 23, No. 4 (2001), pp. 113-25.

It is respectfully submitted that these documents show that infection with various *Mycobacterium* spp. induces production of IL-10 in a host. And it is respectfully submitted that the examples in the above-identified application prove that neutralization of IL-10 activity removes the above immuno suppression so as to diagnose infection with *Mycobacterium* spp. at a high sensitivity, taking paratuberculosis as a typical example. It is respectfully submitted that in view of Applicants' disclosure as originally filed as a whole, to one of ordinary skill in the art,

one of ordinary skill in the art could have applied the present invention to various *Mycobacterium* spp. without undue experimentation. Again, while some experimentation may have been necessary, such experimentation would not be undue.

In view of the foregoing comments and amendments, and in view of the submitted documents, reconsideration and allowance of all presently pending claims, and passing of the above-identified application to issue in due course, are respectfully requested.

To the extent necessary, Applicants hereby petition for an extension of time under 37 CFR 1.136. Kindly charge any shortage of fees due in connection with the filing of this paper, including any extension of time fees, to the Deposit Account of Antonelli, Terry, Stout & Kraus, LLP, Account No. 01-2135 (case 1333.46042X00), and please credit any overpayments to such Deposit Account.

Respectfully submitted,

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Review

Hijacking and exploitation of IL-10 by intracellular pathogens

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Abstract

Macrophages play a central role in infections, as a target for pathogens and in activation of the immune system. Interleukin-10 (IL-10), a cytokine produced by macrophages, is a potent immunosuppressive factor. Some intracellular pathogens specifically target macrophages for infection and use IL-10 to dampen the host immune response and stall their elimination from the host. Certain viruses induce production of cellular IL-10 by macrophages, whereas other viruses encode their own viral IL-10 homologs. Additionally, specific bacteria, including several *Mycobacteria* spp. and *Listeria monocytogenes*, can survive and replicate in macrophages while inducing cellular IL-10, highlighting a potential role for IL-10 of macrophage origin in the immunosuppressive etiology of these pathogens. Thus, the exploitation of IL-10 appears to be a common mechanism of IMMUNOSUPPRESSION by a diverse group of intracellular pathogens that can infect macrophages.

Author Keywords: Macrophages; IL-10; Mycobacteria spp.; Listeria monocytogenes; Anti-inflammatory; MHC II

Article Outline

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Endogenous Inhibition of Antimycobacterial Immunity by IL-10 Varies between Mycobacterial Species

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Roach DR, Martin E, Bean AGD, Rennick DM, Briscoe H, Britton WJ. Endogenous Inhibition of Antimycobacterial Immunity by IL-10 Varies between Mycobacterial Species. Scand J Immunol 2001;54:163–170

Interleukin (IL)-10 is an immunoregulatory cytokine that inhibits both Th1-like T cell responses and macrophage activation. Deficiency of IL-10 has been associated with increased Th1-like $CD4^+$ T-cell responses and increased clearance of some intracellular pathogens, however, its role in mycobacterial infections is controversial. In order to examine the effects of mycobacterial virulence on the outcome of infection we compared infection with *Mycobacterium avium* and virulent *Mycobacterium tuberculosis* in C57Bl/6 IL-$10^{-/-}$ mice. *M. avium* infection in IL-$10^{-/-}$ mice resulted in sustained increases in interferon (IFN)-γ-secreting T-cell responses and was associated with the increased clearance of *M. avium* from the liver and lung. By contrast, *M. tuberculosis* infection in IL-$10^{-/-}$ mice led to a transient increase in IFN-γ T-cell responses at 4 weeks postinfection, with reduced bacterial burden in the lungs. This was not sustained so that by 8 weeks there was no difference to wild-type (WT) mice. *In vitro* infection of IL-$10^{-/-}$ macrophages with *M. avium*, but not *M. tuberculosis*, led to an increased IL-12 production. Therefore, endogenous IL-10 exerts a significant inhibition on specific IFN-γ T-cell responses to *M. avium* infection, however, this effect is short lived during the *M. tuberculosis* infection, and fails to influence the long-term course of infection.

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INTRODUCTION

The control of mycobacterial infections requires the activation of antigen-specific $CD4^+$ T cells which are recruited back to the site of infection where they stimulate infected macrophages to kill the mycobacteria [1]. The macrophage activating cytokine critical to this process is IFN-γ [2], but others are also required to control the infection, including tumour necrosis factor (TNF) [3] and lymphotoxin-α (LTα) [4]. TNF synergises with IFN-γ to induce maximal activation of macrophages [5], while both TNF and LTα are required for granuloma formation to contain the infection [3, 4]. IL-10 is a pleiotropic cytokine with a broad range of immunoregulatory activities [6], which could influence the outcome of mycobacterial infections. First, IL-10 inhibits the development, proliferation and cytokine production of Th1-like T cells, principally through its inhibitory effects on macrophages and dendritic cells (DC) [6, 7]. These effects were initially characterized with murine IFN-γ secreting Th1-like T-cell clones, however, IL-10 was subsequently found to inhibit cytokine production from both Th1-like and Th2-like human T cells [8]. Secondly, IL-10 has direct inhibitory effects on the macrophage activation with reductions in the expression of cell-surface molecules [9], a decreased production of pro-inflammatory cytokines, such as TNF and IL-1β [10], and reduced responses to activation signals [11]. For example, IL-10 inhibits an IFN-γ-induced activation of inducible nitric oxide (NO) synthase and the production of reactive nitrogen intermediates [9]. As a result exogenous IL-10 blocks IFN-γ-stimulated growth inhibition of bacille Calmette–Guérin (BCG) in macrophages [12]. Deletion of

¶ These authors contributed equally to this study.

the IL-10 gene in mice resulted in the expansion of IFN-γ-secreting Th1-like $CD4^+$ T cells and inflammatory pathology, such as chronic enterocolitis [13, 14]. Infection of IL-10-deficient ($IL\text{-}10^{-/-}$) mice with the intracellular pathogens, *Listeria monocytogenes* [15] and *Chlamydia trachomatis* [16] resulted in enhanced IFN-γ T-cell responses and increased the clearance of these organisms. However, the increased $CD4^+$ T-cell responses of $IL\text{-}10^{-/-}$ mice to other pathogens, such as *Toxoplasma gondii* [17] and *Typanosoma cruzi* [18] resulted in an excessive production of IFN-γ and pro-inflammatory cytokines, exuberant cellular inflammation and death.

In the case of mycobacterial infections, there is conflicting evidence as to the role of endogenous IL-10. Neutralization experiments with anti-IL-10 antibodies demonstrated an enhanced resistance to the *M. avium* infection [19, 20] although the mechanism of this activity was not determined. Initial reports with IL-10 gene deficient mice showed no difference in the course of *M. bovis* (BCG) [21] and *M. tuberculosis* infection [22]. Subsequently, more detailed studies revealed an increased resistance to the BCG infection in $IL\text{-}10^{-/-}$ mice early in the course of infection [23, 24]. We considered that these differences may, in part, reflect the virulence of the mycobacterial species examined. Members of the *M. avium* complex usually cause progressive disease in humans with advanced CD4 T-cell deficiency, as seen in HIV/AIDS [25] or those with defects in IFN-γ signalling or production [26]. By contrast, *M. tuberculosis* is more virulent and causes a progressive infection in apparently normal subjects or early in the course of HIV/AIDS during mild immunodeficiency. Therefore, we have compared the effect of an endogenous IL-10 deficiency on the pattern of Th1 T-cell responses and the clearance of mycobacteria during infection with either *M. avium* or the more virulent *M. tuberculosis*. There was a sustained increase in the frequency of IFN-γ-secreting Th1-like T cells and clearance of the mycobacteria in *M. avium* infected $IL\text{-}10^{-/-}$ mice, compared to WT C576BL/6 mice indicating that endogenous IL-10 has a significant immunoregulatory role during *M. avium* infection. Following the *M. tuberculosis* infection, there was a transient increase in Th1-like T-cell responses at 4 weeks postinfection that was associated with a significant reduction in the bacterial load at this early time point. This effect, however, was not sustained in the lung, indicating that IL-10 does not play a major regulatory role in chronic *M. tuberculosis* infection.

METHODS

Mice. The control WT mice were 6–8-weeks-old C57Bl/6 mice obtained from the Animal Resource Center (Perth, Australia). IL-10 gene deficient mice ($IL\text{-}10^{-/-}$) were generated as previously described [13] and back-crossed 10 times onto a C57Bl/6 background. All mice were housed under specific pathogen-free conditions in the Centenary Institute animal facility until infection, when they were transferred either to a level 2 physical containment facility following *M. avium* infection or a level 3 physical containment facility following *M. tuberculosis* infection.

Bacteria and experimental infections. The *M. avium* strain (serotype 8) utilized causes a chronic infection in C57Bl/6 mice [27] and was kindly provided by C. Cheers (University of Melbourne, Victoria, Australia). It was grown in supplemented Middlebrook 7H9 liquid medium (Difco, Detroit, USA) for 7 days at 37 °C and then stored at −70 °C. Before use the inoculum was sonicated for 10 s to disperse clumps. Mice were infected intravenously through the lateral tail vein with 1×10^6 cfu of *M. avium*. A Middlebrook airborne infection apparatus (Glas-col, Terre Haute, IN, USA) was used to infect mice with 100 cfu of *M. tuberculosis* H37Rv, as previously described [3]. The numbers of viable bacteria in target organs were examined over time by plating serial dilutions of whole organ homogenates on supplemented Middlebrook 7H11 agar (Difco) and counting bacterial colony formation after incubation at 37 °C for 14 days for *M. avium* infections or 21 days for *M. tuberculosis* infections.

T-cell responses to mycobacterial antigens. Spleens from *M. avium*-infected or mediastinal lymph nodes (MLN) from *M. tuberculosis*-infected $IL\text{-}10^{-/-}$ and WT mice were removed and single-cell suspensions were prepared. Erythrocytes were lysed in a hypotonic ammonium chloride lysis buffer and the remaining cells were washed, counted and suspended in complete RPMI media [RPMI 1640 (Cytosystem, Sydney, Australia) with 10% foetal bovine serum (FCS) (Trace, Sydney, Australia), 2 mM L-glutamine (Sigma), 10 mM HEPES (Sigma), 10 mM Na_2CO_3, 0.5 μM 2-mercaptoethanol (Sigma), 100 U/ml penicillin (Trace) and 100 μg/ml streptomycin (CSL, Melbourne, Australia). To measure antigen-specific T-cell responses, splenocytes from *M. avium*-infected cells were cultured in the presence of *M. avium* sonicate (10 μg/ml), and MLN cells from *M. tuberculosis*-infected mice with purified protein derivative (PPD) of *M. tuberculosis* (Statens seruminstitut, Copenhagen, Denmark). Lymphocyte proliferation and cytokine assays for IFN-γ were performed as described previously [28, 29]. For proliferative responses, the cells were pulsed with 1 μCi of ^{3}H-thymidine (NEN Life Sciences, Boston, MA, USA) for the final 6 h of culture and then harvested onto glass fibre filters. The incorporated ^{3}H-thymidine was determined by liquid scintillation spectroscopy (Pharmacia/Wallace Oy, Turku, Finland). Specific ^{3}H-thymidine incorporation was calculated by subtracting the mean counts per minute (cpm) in unstimulated wells from the mean cpm of test samples. The concentration of IFN-γ in culture supernants was determined using a capture ELISA. In order to determine the frequency of IFN-γ producing cells, spleen or MLN cells were cultured in Multiscreen 96-well filtration plates (Millipore, Bedford MA, USA) for 16 h in the presence of the appropriate stimulus or medium alone and ELIspots developed as previously described [29].

Phenotypic analysis of pulmonary and splenic infiltrates. Lung cell homogenates from $IL\text{-}10^{-/-}$ and WT mice following M. tuberculosis infection were prepared as previously described [3]. Spleen cell suspensions from $IL\text{-}10^{-/-}$ and WT mice following *M. avium* infection were prepared as for T-cell responses. The following monoclonal antibodies (MoAb) were used: CD4 (CT-CD4; Caltag, San Francisco, CA, USA), CD8 (CT-CD8; Caltag) and CD45R(B220)(RA3–6B2; Caltag). The staining of cells was performed as previously described [3] and analyzed on a FACScan (Becton Dickinson, San Jose, CA, USA).

Il-12 production from adherent splenocytes. Spleens from uninfected WT and $IL\text{-}10^{-/-}$ mice were removed and single-cell suspensions were prepared. Erythrocytes were lysed in a hypotonic ammonium chloride lysis buffer and the remaining cells were washed, counted and suspended in complete RPMI medium without antibiotics. Cells were plated at 1×10^6 cells per well in 24-well tissue culture plates and incubated for 16 h. The nonadherent cells were removed and adherent

macrophages were infected with either *M. avium* or *M. tuberculosis* H37Rv (MOI 1 : 1). After 4 h the cells were washed to remove extracellular bacteria, and then cultured for 48 h at 37 °C, at which time the supernatants were frozen. The IL-12 production was measured using the OptEIA set mouse IL-12 (p70) capture ELISA (Pharmingen).

Statistical analysis. Where appropriate the differences between WT and IL-$10^{-/-}$ were tested for statistical significance by unpaired Student's *t*-tests. Cfu values were subject to $\log_{10}$ transformation before analysis.

RESULTS

IL-$10^{-/-}$ mice display increased resistance to M. avium infection

To examine the effect of IL-10 deficiency on resistance to *M. avium*, WT and IL-$10^{-/-}$ were infected intravenously with 1×10^6 cfu of *M. avium* and the course of infection was followed over time. IL-10-deficient mice had significantly reduced bacterial loads in their lungs over the 12-week course of infection (Fig. 1A) when compared to WT controls. In the liver, IL-$10^{-/-}$ and WT mice had similar bacterial loads at week 2 postinfection, but by week 4 there were significantly reduced bacterial numbers in the IL-$10^{-/-}$ mice. This reduction in bacterial load persisted over the course of the infection, such that at week 12 there were approximately 10-fold fewer bacteria in the livers of IL-$10^{-/-}$ mice compared to WT controls.

IL-$10^{-/-}$ mice exhibit increased Th1-like T-cell responses to M. avium

Phenotypic analysis of splenic lymphocytes following *M. avium* infection showed similar numbers of $CD4^+$ and $CD8^+$ T cells in both WT and IL-$10^{-/-}$ following infection with *M. avium* (data not shown). To determine if the increased resistance of IL-$10^{-/-}$ mice to infection was associated with a more robust Th1-like T-cell response, T-cell responses were examined over the course of *M. avium* infection in both WT and IL-$10^{-/-}$ mice. Splenocytes from *M. avium*-infected IL-$10^{-/-}$ mice exhibited stronger proliferative responses when cultured in the presence of *M. avium* sonicate (Table 1). Furthermore, IL-$10^{-/-}$ mice had significantly more *M. avium*-specific IFN-γ producing T cells in their spleens over the 12-week course of the infection (Fig. 2A), and this resulted in increased IFN-γ release on *M. avium* stimulation (Fig. 2B).

IL-$10^{-/-}$ mice display transient increased resistance to M. tuberculosis infection

To examine the effect of endogenous IL-10 on *M. tuberculosis* infection, WT and IL-$10^{-/-}$ mice were exposed to an aerosol of *M. tuberculosis* H37Rv and the course of infection monitored over time. At 4 weeks postinfection there were significantly fewer mycobacteria in the lungs of IL-$10^{-/-}$ mice compared to the WT controls (Fig. 3A). This increased resistance to infection was not maintained, so that by 8 weeks postinfection equivalent

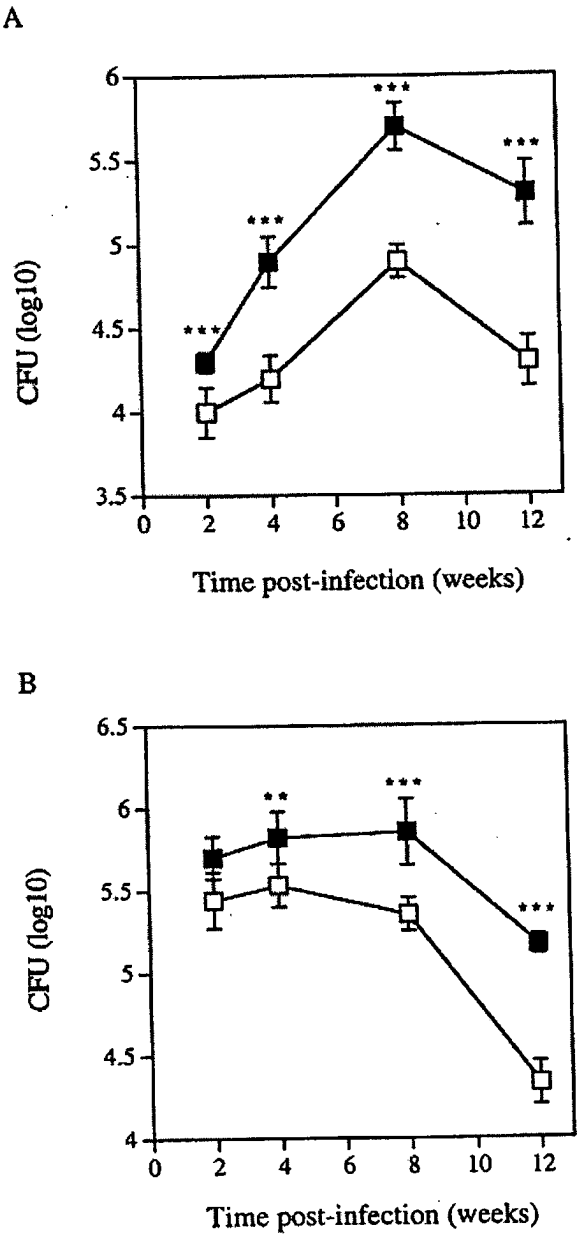


Fig. 1. Interleukin (IL)-10-deficient mice exhibit decreased bacterial loads following infection with *M. avium*. Wild-type (WT) (■) and IL-$10^{-/-}$ (□) mice were infected with 1×10^6 *M. avium* intravenously and the numbers of viable bacteria present in both the lung (A) and liver (B) were determined over time. The data points represent the mean ± SEM of the colony-forming units (cfu) from 5 WT and 5 IL-$10^{-/-}$ mice at each time-point from 1 of 2 representative experiments. Statistical differences were determined by unpaired Student's *t*-tests: ** $P < 0.01$, *** $P < 0.001$.

Table 1. Proliferative responses of splenocytes in WT and IL-$10^{-/-}$ mice following infection with *M. avium*

Time postinfection (weeks)	WT†	IL-$10^{-/-}$†
2	16089 (2100)	28885 (2558)*
4	22714 (2987)	49091 (2224)**
8	20639 (2112)	51979 (2554)***
12	14607 (1447)	29499 (2960)***

†The mean (± SEM) of specific ^{3}H-thymidine incorporation from splenocytes from 5 WT and 5 IL-10 deficient animals at each time point. The significance of the differences between WT and IL-$10^{-/-}$ mice at each time point were tested by Student's *t*-tests (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

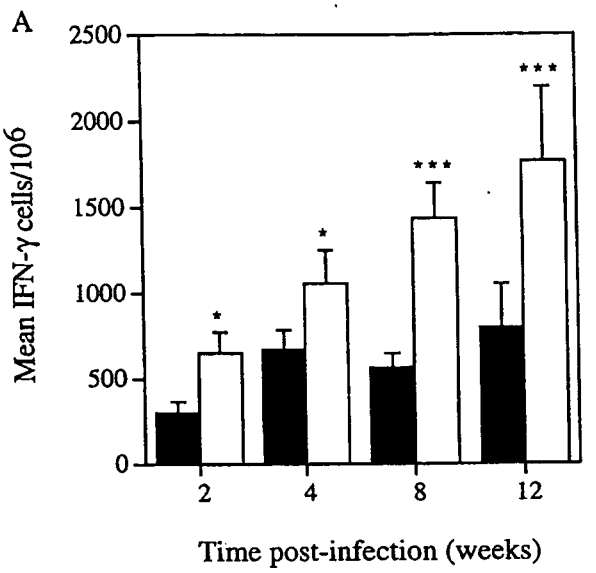
numbers of mycobacteria were recovered from the lungs of both IL-$10^{-/-}$ and WT mice. In contrast, the spleens of IL-$10^{-/-}$ mice contained significantly fewer mycobacteria at weeks 8 and 12 postinfection compared to WT mice (Fig. 3B).

IL-$10^{-/-}$ mice exhibit transiently increased T-cell responses to M. tuberculosis

There were no differences in the pattern of recruitment of $CD4^+$ and $CD8^+$ T cells, as well as B cells, to the lung following *M. tuberculosis* infection between WT and IL-$10^{-/-}$ mice (data not shown). To determine if the transiently increased resistance to *M. tuberculosis* infection was associated with increased T-cell responses, the frequency of antigen-specific IFN-γ-producing cells and production of IFN-γ in the mediastinal lymph nodes draining the lungs cells was examined. At 4 weeks postinfection, there were significantly more PPD-specific IFN-γ-producing cells (Fig. 4A) in the mediastinal lymph nodes of IL-$10^{-/-}$ mice when compared to WT controls and this was associated with a significantly increased IFN-γ release (Fig. 4B). This difference diminished over time, such that at 8 and 12 weeks postinfection equivalent T-cell responses were seen in WT and IL-$10^{-/-}$ mice.

Increased production of IL-12 in IL-$10^{-/-}$ splenocytes following M. avium infection

The development of a Th1-like immune response is dependent on IL-12, and IL-10 may downregulate IL-12 production. Adherent macrophages from the spleens of WT and IL-$10^{-/-}$ mice were infected with *M. avium* and *M. tuberculosis* and the release of IL-12 was measured 48 h postinfection. Both *M. avium* and *M. tuberculosis* induced an IL-12 production in WT macrophages. Macrophages from IL-$10^{-/-}$ mice produced significantly more IL-12 following *M. avium* infection, but not following *M. tuberculosis*, when compared to WT infected cells (Fig. 5).



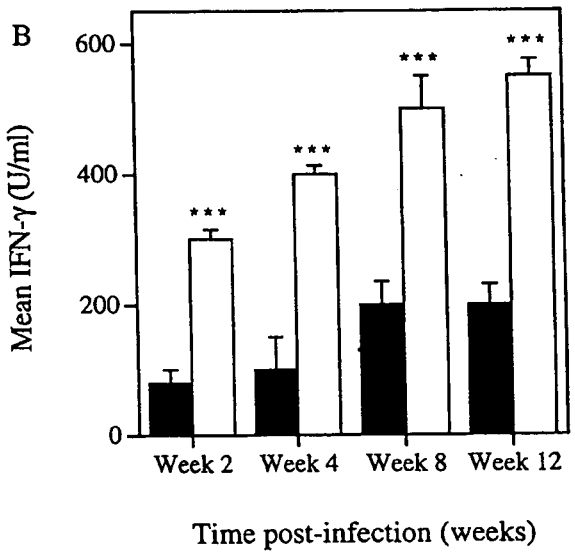


Fig. 2. IL-$10^{-/-}$ mice exhibit increased Th1-like T-cell responses to *M. avium*. Splenocytes from WT (solid bars) and IL-$10^{-/-}$ (open bars) were cultured in the presence of *M. avium* sonicate or medium alone and the frequency of interferon (IFN)-γ-producing cells (A) and bulk production of IFN-γ (B) were measured over the course of the infection. To determine the antigen-specific response, values from cells cultured with medium alone were subtracted from those cultured with *M. avium* sonicate. The data points represent the mean ± SEM of 5 WT and 5 IL-$10^{-/-}$ mice at each time-point from 1 of 3 representative experiments. Statistical differences were determined by unpaired Student's *t*-tests: * $P < 0.05$, *** $P < 0.001$.

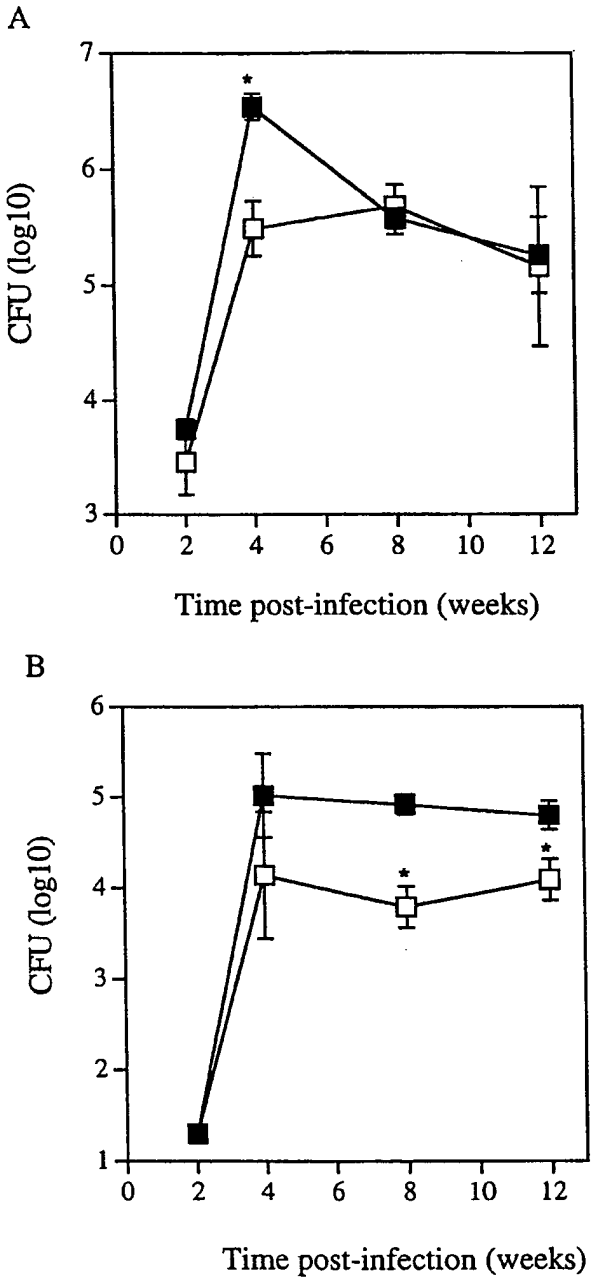


Fig. 3. IL-10-deficient mice display transiently increased resistance to *M. tuberculosis* infection. WT (■) and IL-$10^{-/-}$ (□) mice were infected with 100 cfu of *M. tuberculosis* aerosol and the numbers of viable bacteria present in both the lung (A) and spleen (B) were determined over time. The data points represent the mean ± SEM of the cfu from 5 WT and 5 IL-$10^{-/-}$ mice at each time point. Statistical differences were determined by unpaired Student's *t*-tests: * $P < 0.05$.

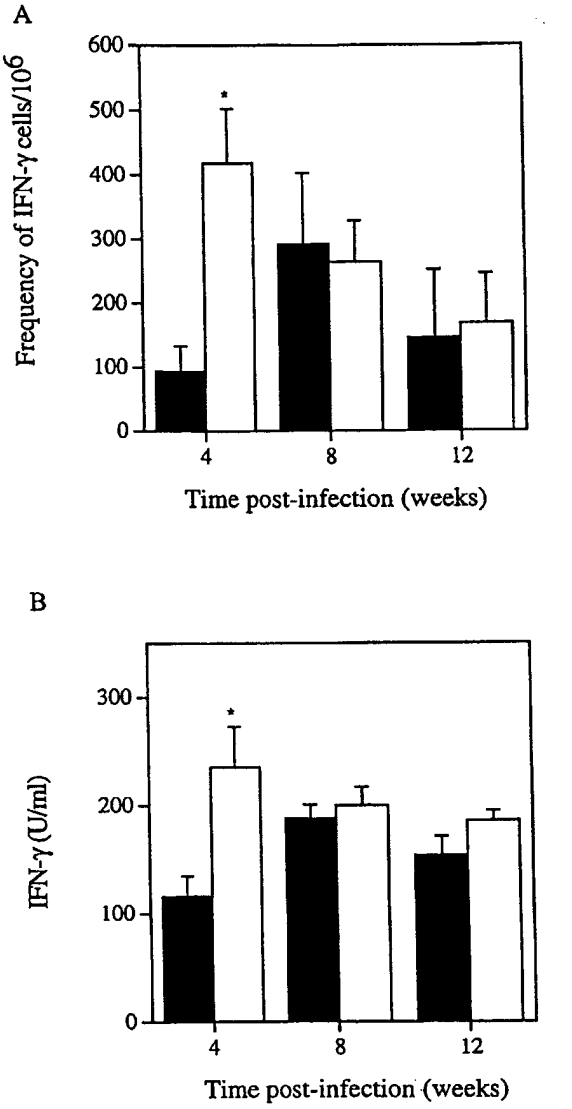


Fig. 4. IL-10-deficient mice exhibit transiently increased Th1-like T-cell responses following infection with *M. tuberculosis*. MLN from WT (solid bars) and IL-$10^{-/-}$ (open bars) were cultured in the presence of PPD or medium alone and the frequency of IFN-γ-producing cells (A) and bulk production of IFN-γ (B) were measured over the course of infection. To determine the antigen-specific response, values from cells cultured with medium alone were subtracted from those cultured with PPD. The data points represent the mean ± SEM of 5 WT and 5 IL-$10^{-/-}$ mice at each time point. Statistical differences were determined by unpaired Student's *t*-tests: * $P < 0.05$.

DISCUSSION

There were significant differences in the impact of IL-10 on infection with these two species of mycobacteria. In the case of *M. avium* infection, deficiency in the endogenous IL-10 production resulted in a sustained increase in Th1-like T-cell

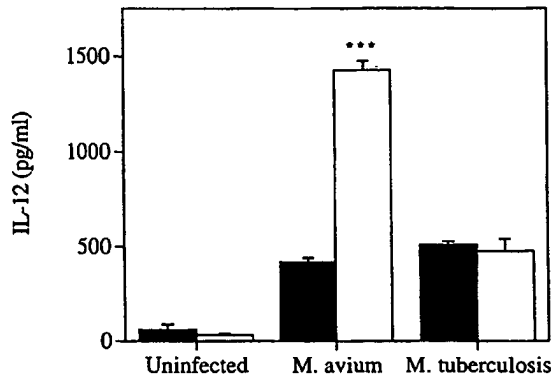


Fig. 5. Increased production of IL-12 in IL-$10^{-/-}$ adherent splenocytes following infection with *M. avium*. Adherent cells from uninfected spleens of WT (closed bars) and IL-$10^{-/-}$ (open bars) were infected with *M. avium* or *M. tuberculosis* (MOI 1 : 1) and 48 h postinfection the IL-12 concentration was determined by ELISA. The data points represent the mean ± SEM of triplicate wells. Statistical differences were determined by unpaired Student's *t*-tests: *** $P < 0.001$.

responses, as manifested by an increased frequency of antigen-specific IFN-γ-secreting T cells, and increased resistance to infection in both the liver, the major site of *M. avium* growth after intravenous infection, and the lung. Macrophages from IL-$10^{-/-}$ mice showed an increased IL-12 production and this may contribute to the induction and maintenance of an expanded pool of helper T cells to *M. avium*. By contrast, the effect of IL-10 deficiency upon aerosol infection of the lung with *M. tuberculosis* was less marked. There was a significant increase in the frequency of antigen-specific IFN-γ-secreting T cells in mediastinal lymph nodes at 4 weeks when the infection peaks in the lung, and this was associated with a modest reduction of the bacterial burden at that time point. Thereafter there was no difference in the lung, although the difference in bacterial load persisted in the spleen, a site of dissemination for *M. tuberculosis* infection.

Members of the *M. avium* complex are usually only significant human pathogens when $CD4^+$ T-cell immunity is markedly reduced [25], and there is considerable variation in the virulence of different *M. avium* isolates in mice. Infection with the *M. avium* strain tested here is restrained by IFN-γ responses for more than 6 months [27] and the infection only progresses late when the *M. avium* specific $CD4^+$ T-cell population wanes through apoptosis [30]. The inhibitory effects of IL-10 on the development and expression of T-cell immunity were evident from 2 weeks of *M. avium* infection and increased until 12 weeks. Previous studies with neutralizing anti-IL-10 antibodies revealed inhibitory effects of IL-10 on resistance to infection with two different strains of *M. avium* [19, 20] although these studies did not determine whether the effect of IL-10 was caused by limiting the $CD4^+$ Th1-like T-cell population or by macrophage deactivation. The increased antigen-specific IFN-γ T-cell responses clearly contributed to the increased resistance to *M. avium* infection in the absence of endogenous IL-10 (Fig. 2). Neutralization of IL-10 enhanced immunity to other intracellular bacterial pathogens, as evidenced by the increased clearance of *Salmonella cholerausis* [31] and *Brucella abortus* [32] infections in mice. In both cases there was evidence of increased IFN-γ responses in cells from infected tissues and increased expansion of γδ T cells during the *Salmonella* infection. The downregulatory effect of IL-10 on Th1-like T-cell responses was demonstrated by the infection of IL-$10^{-/-}$ mice with other intracellular pathogens. Both innate and acquired immunity to *Listeria monocytogenes* were increased in IL-$10^{-/-}$ deficient mice [15]. During primary infection there was an increased production of pro-inflammatory cytokines and a more marked Th1 polarization in IL-$10^{-/-}$ mice compared to WT mice. Importantly, IL-$10^{-/-}$ mice were more resistant to secondary *Listeria* infection with maintenance of enhanced IFN-γ $CD4^+$ and $CD8^+$ T-cell responses. The clearance of *Chlamydia trachomatis* was also significantly accelerated in C57BL/6 IL-$10^{-/-}$ mice compared to WT animals, and this was associated with stronger and more persistent IFN-γ production from helper T cells and stronger DTH responses [16]. These effects were reversed by the administration of the exogenous IL-10. Therefore, IL-10 inhibits the induction and expansion of Th1-like T-cell responses to a range of intracellular pathogens as well as *M. avium*. This does not rule out IL-10 having additional inhibitory effects on IFN-γ-mediated activation of macrophages and the significance of this may varying with infecting pathogen.

DC are the critical antigen presenting cell (APC) for the induction of $CD4^+$ T-cell immunity and are likely to be the site of action for endogenous inhibitory effects of IL-10 on the development of Th1-like T-cell responses. Infection of immature murine DC with BCG stimulates the early transcription of both IL-12 and IL-10 mRNA suggesting the balance of these two cytokines is important in the pattern of the ensuing $CD4^+$ T-cell response [33]. When DC from IL-$10^{-/-}$ mice were infected with BCG, there was a significant increase in IL-12 production, and this was further increased by ligation of CD40 on the IL-$10^{-/-}$ DC [34]. Inhibition of IL-10 signalling *in vivo* also biases the pattern of immune responses to a Th1-like phenotype. Treatment with antibodies against the IL-10 receptor during immunization with ovalbumin (OVA) resulted in enhanced IFN-γ-secreting Th1 T-cell responses [35].

The removal of endogenous IL-10 had less effect on the progress of infection with more virulent *M. tuberculosis*, particularly in the lung. Transiently increased resistance was associated with a significant increase in IFN-γ-secreting T cells, however, this enhanced T-cell immunity was not sustained. In another study of *M. tuberculosis* infection in IL-10-deficient mice, there were no differences in pulmonary bacterial load at day 28 postinfection, however, T-cell responses to mycobacterial antigens were not measured [22]. One possible explanation for the difference between the *M. avium* and *M. tuberculosis* infection in the IL-$10^{-/-}$ mice is that the more virulent *M. tuberculosis* infection may stimulate a wider range of host immunosuppressive mechanisms that may compensate for the

lack of endogenous IL-10. For example, *M. tuberculosis* infection stimulates the production of transforming growth factor-β (TGF-β) which is strongly expressed by alveolar macrophages [36]. The reduced T-cell responses to *M. tuberculosis* evident in human subjects with active tuberculosis are predominantly owing to the production of TGF-β [37] and this is likely to be active in the lungs of *M. tuberculosis*-infected mice. Furthermore, the balance between IL-12 and IL-10 in the induction of different patterns of $CD4^+$ T-cell responses may be influenced by multiple factors, including the route and the dose of exposure to the pathogen and the genetic background of the host. Different patterns of cellular infiltrate and cytokine production were observed in IL-$10^{-/-}$ mice infected with *Aspergillus fumigatus* dependant on whether the initial sensitization was by the intranasal or systemic route and on the background strain of the gene-deficient mice [38]. The engagement of different inhibitory mechanisms appeared able to compensate for the absence of the inhibitory effects of IL-10 on the recruitment and activation of $CD4^+$ T cells to the lung. In the case of mycobacterial infection, the marked differences in the host response to *M. avium* and *M. tuberculosis* infection both *in vivo* and *in vitro* in the absence of IL-10 suggests that this is owing to differences in the infecting mycobacterial species.

Two out of three previous studies showed comparable enhanced resistance of IL-$10^{-/-}$ mice early in infection with BCG [21, 23, 24]. At 2 weeks there was an increased clearance of BCG from the liver and spleen following intravenous or intraperitoneal infection. However, paradoxically different patterns of granuloma formation were observed, with intraperitoneal infection leading to fewer granulomas in IL-$10^{-/-}$ mice [23] and intravenous BCG infection generating larger hepatic granulomas but enhanced clearance of bacteria [24]. The dominant effect of the removal of the IL-10 on BCG infection appeared to be an enhanced macrophage activation independent of the amount of IFN-γ produced [23]. This was supported by small but significant increases in NO and prostaglandin-E2 production by activated peritoneal exudate cells from IL-10-deficient mice [24]. When transgenic mice that secreted IL-10 from their T-cell compartment were infected with BCG, there was a direct inhibition of macrophage activation with no reduction in IFN-γ T-cell responses, and this resulted in increased bacterial growth [39]. This supports the hypothesis that IL-10 influences mycobacterial infection through direct macrophage deactivating effects. Therefore, IL-10 can influence the resistance to intracellular pathogens both by inhibiting the development of IFN-γ-secreting Th1-like $CD4^+$ T-cell responses and by direct effects on macrophages, either through blocking their IFN-γ-mediated activation, or by inhibiting the release of pro-inflammatory cytokines. Recently, a further mechanism for IL-10 s 'anti-inflammatory' activity was revealed when IL-10 was found to de-link the signalling between chemokine and chemokine receptors within inflammatory tissues [40]. This resulted in the production of functional chemokine decoy receptors that were unable to elicit migration of DC.

The importance of the immunoregulatory role of IL-10 may vary for the particular pathogen and the site of infection. The increased inflammatory response to the *Toxoplasma gondii* and *Typanosoma cruzi* infection resulted in marked cellular inflammation and death, rather than increased resistance to infection [17, 18]. Therefore, the value of inhibiting IL-10 responses to increase the resistance to individual intracellular pathogens should be examined with each individual pathogen.

ACKNOWLEDGMENTS

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Enhanced immune response in Mycobacterium bovis bacille calmette guerin (BCG)-infected IL-10-deficient mice.

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The role of the endogenous interleukin-10 (IL-10) in the control of Mycobacterium bovis Bacille Calmette Guerin (BCG) infection was assessed using IL-10-deficient (IL-10-/-) mice. Similar to wild-type (WT) mice, IL-10-/- mice were resistant to intravenous challenge with Mycobacterium bovis BCG. Significantly higher plasma concentrations of IL-12 and tumour necrosis factor (TNF) indicated an elevated protective immune response of IL-10-/- mice. Determination of bacilli burden in IL-10-/- mice showed accelerated clearance in the lungs, spleen and the liver in comparison to WT mice. Enhanced inflammation and a vigorous granulomatous response accompanied accelerated mycobacterial clearance. Immunohistochemical analysis of hepatic granulomas from IL-10-/- mice revealed augmented lymphocyte recruitment and macrophage activation, such as increased major histocompatibility complex (MHC) class II and inducible nitric oxide synthase (iNOS) expression. Further, it was found that enlarged granulomas persisted subsequent to mycobacterial clearance and failed to resolve in the absence of IL-10. In conclusion, endogenous IL-10 dampens the cell-mediated immune response to mycobacterial infection.

PMID: 12435106 [PubMed - indexed for MEDLINE]

Related Articles

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[J Immunol. 1998]

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Profiles of IFN-γ and its regulatory cytokines (IL-12, IL-18 and IL-10) in peripheral blood mononuclear cells from patients with multidrug-resistant tuberculosis

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SUMMARY

This study investigated the profiles of IFN-γ and its regulatory cytokines (IL-12, IL-18 and IL-10) in response to a purified protein derivative (PPD) antigen in peripheral blood mononuclear cells (PBMC) from 18 HIV-negative patients with multidrug-resistant tuberculosis (MDRTB), and compared them with those from 19 healthy tuberculin reactors (HTR). ELISA results showed that following stimulation with PPD, IFN-γ production was significantly reduced, whereas production of both IL-18 and IL-10 was significantly elevated in MDRTB patients compared with HTR. Three out of 18 patients with MDRTB of greater than 4 years duration showed significantly elevated IL-12 p70 production, induced by *in vitro* PPD stimulation of their PBMC, when compared with data from HTR. However, when taken as a group, MDRTB patients were similar to HTR in their IL-12 p70-producing capacity. IL-12 p70 protein paralleled IL-12 p40 protein expression. In addition, the production of IL-12 p40 was significantly correlated with IL-10 in all patients, but was not correlated with IFN-γ. Neutralization of IL-10 increased IL-12 p40 about twofold, but did not significantly alter IFN-γ induction in MDRTB. IFN-γ in MDRTB was highly correlated with lymphoproliferation and CD4 counts, but was not correlated with IL-12, IL-18 or IL-10 production. Our findings suggest that patients with MDRTB have dysregulated IL-12, IL-18 and IL-10 production during *Mycobacterium tuberculosis* infection, and the cytokine profiles are similar to those in patients with drug-sensitive advanced TB previously reported in the literature. In addition, IL-10 may not have a dominant role in defective IFN-γ production in patients with MDRTB.

Keywords multidrug-resistant pulmonary tuberculosis interferon-gamma interleukin-12 interleukin-18 interleukin-10 PPD antigen

INTRODUCTION

Multidrug-resistant tuberculosis (MDRTB) is a significant clinical problem that is associated with high morbidity and mortality, and long-term survival for infected immunocompetent patients is reported to be about 70% [1]. The increased incidence of MDRTB is a significant public health and therapeutic problem that may quickly worsen as the HIV epidemic spreads. In addition, it has been reported that outbreaks of MDRTB are a serious clinical problem among individuals who are HIV-negative [2].

MDRTB is often accompanied by host immunosuppression; consequently, immune response enhancement may be a useful supplement to conventional MDRTB chemotherapy [3]. $CD4^+$ T cells are involved in anti-mycobacterial activity [4], and interferon (IFN)-γ produced by $CD4^+$ T cells plays a critical role in protective immunity [5–7]. In HIV-positive TB patients, the CD4 count is the sole independent predictor of survival [8]. In HIV-negative patients with MDRTB, CD4 lymphocytopenia appears to correlate with the severity of disease and low IL-2/IFN-γ production in response to *Mycobacterium tuberculosis* and purified protein derivatives (PPD) [9].

Although there is substantial evidence to support a role for cell-mediated immunity in the protective immune response against TB [10], little is known about Th1 regulatory cytokine production in MDRTB. Diverse cytokines, including interleukin (IL)-2, IL-12 and IL-18, are known to play important roles in anti-TB cell-mediated immunity. Two cytokines, IL-12 and IL-18, are currently regarded as the primary inducers of IFN-γ production in inflammatory reactions [11,12]. IL-12 is an inducible, heterodimeric, disulphide-linked cytokine that is composed of 35-

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and 40-kD subunits encoded by separate genes [13]. IL-12 is the most important cytokine for directing primary Th1 differentiation in $CD4^+$ T cells *in vitro* and *in vivo*. Studies have shown that treating mice with IL-12 at the time of TB infection results in increased resistance to infection, illustrating the importance of IL-12 for protective immunity against TB [14,15].

IL-18, initially described as an IFN-γ-inducing factor [16], is a multi-functional cytokine that is produced by a wide variety of cells, including mononuclear phagocytes [17]. IL-18 acts together with IL-12 as an early signal in the development of Th1 responses [11,18]. However, IL-18 alone does not induce IFN-γ production by T lymphocytes [19]. The presence of secondary stimulants, particularly IL-12 or microbial agents, is required for IL-18-induced IFN-γ production [20]. Active pulmonary TB is associated with the enhanced production and activity of immunosuppressive molecules, such as IL-10 and transforming growth factor (TGF)-β1 [21]. IL-10 and TGF-β1 have many overlapping biological effects, including T-cell suppression, macrophage deactivation, modulation of pro-inflammatory cytokines and interference with antigen-presenting cell function [22,23].

This study investigated the PPD-induced production of IL-12, IL-18 and IL-10 in peripheral blood mononuclear cells (PBMC) from patients with MDRTB. We found that IL-18 and IL-10 production was significantly up-regulated after PPD stimulation by PBMC from MDRTB patients, whereas IFN-γ production was greatly reduced compared with production in human tuberculin reactors (HTR). In addition, PPD-induced IL-12 production was highly correlated with IL-10 production in MDRTB, but not with either IFN-γ or IL-18 production.

MATERIALS AND METHODS

Subjects

Patients and healthy volunteers consented to take part in this study. Whole blood was obtained by venipuncture from 18 HIV-negative patients with culture-proven MDRTB at the National Mokpo Tuberculosis Hospital (Mokpo, Chonnam, Korea). These patients were at various stages in their clinical course, and had different treatment durations; minimally, all patients showed resistance to rifampicin and isoniazid. A complete history was taken and a physical examination was performed on each patient by one of the investigators. All patients but one (C3) had remained infected for over 2 years, despite anti-tuberculosis drug therapy. The patients (four women and 14 men) ranged in age from 23 to 73 years, with a mean age of 41·7 years (s.d. = 16). All were classified as having far advanced disease, with massive involvement and multiple cavities (total diameter greater than 4 cm), when assessed by X-ray. The patient profiles are shown in Table 1.

Nineteen HTR exhibited skin reactions of more than 15 mm after an intradermal test with 5 units of PPD-RT23 (Statens Seruminstitut, Copenhagen, Denmark), within 1–3 years of their PPD skin test examination, and had no previous history of clinical TB. Each of these healthy controls had received *Mycobacterium bovis* Bacille bilié de Calmette-Guerin (BCG) vaccinations as children.

Antigen and antibodies

Tuberculin PPD for *in vitro* assay was purchased from the Statens Seruminstitut and was used at a final concentration of 1·0 μg/ml.

Table 1. Profile of the study group

Number	Sex/Age	Culture severity	BMI*	ID† (years)	TD‡ (months)	CC¶	IM§	CXR**
C1	M/59	1+	20·9	13·5	82	P	P	FA
C2	M/46	1+	22·9	19·0	36	N	P	FA
C3	M/47	2+	18·0 (M)	1·8	22	P	N	FA
C4	M/25	1+	14·5 (M)	3·0	23	N	P	FA
C5	M/41	3+	18·4 (M)	7·0	43	N	P	FA
C7	M/48	1+	15·0 (M)	3·6	43	P	N	FA
C8	M/29	1+	21·8	13·0	38	N	P	FA
C9	F/35	1+	22·7	4·0	31	N	P	FA
C10	F/23	1+	18·6 (M)	3·5	31	N	P	FA
C11	M/31	3+	21·0	6·5	78	P	N	FA
C12	F/23	3+	19·6 (M)	6·0	46	N	P	FA
C13	M/66	3+	17·2 (M)	4·1	49	P	N	FA
C14	M/28	4+	17·0 (M)	5·0	34	N	P	FA
C15	M/73	2+	18·6 (M)	21·0	89	N	P	FA
C17	M/31	3+	23·9	4·5	54	P	N	FA
C18	M/39	1+	19·7 (M)	4·1	49	P	N	FA
C19	F/34	1+	22·0	5·0	32	N	P	FA
C20	M/72	3+	17·0 (M)	6·0	52	P	P	FA

*Body mass index (BMI), body weight/height $(m)^2$; M < 20·0, malnutrition.
†Duration of infection (years), periods with positive cultures.
‡Duration of treatment (months).
¶History of complete cure; P, positive; N, negative.
§Intermittent medication; P, positive; N, negative.
**Severity by chest X-ray; FA, far advanced.

Endotoxin content was measured by *Limulus* amoebocyte lysate assay and was below 1·5 pg/ml in PPD antigen. Neutralizing rat anti-human IL-10 antibodies were purchased from R & D Systems (Minneapolis, MN, USA). For flow cytometric analysis, fluorescein isothiocyanate (FITC)-labelled anti-CD4 and phycoerythrin (PE)-labelled anti-CD8 were purchased from PharMingen (San Diego, CA, USA).

Preparation and stimulation of peripheral blood mononuclear cells

Venous blood was drawn from subjects into sterile blood collection tubes, and PBMC were isolated by density sedimentation over Histopaque-1077 (Sigma, St. Louis, MO, USA). FACS analysis of the PBMC fraction was then performed, and a CD4 count was calculated based on a concomitant complete blood count. Additionally, PBMC were suspended at a density of 1×10^6 viable cells/ml in complete medium [RPMI 1640 (GIBCO-BRL, Gaithersburg, MD, USA) with 10% fetal bovine serum (GIBCO-BRL), sodium pyruvate, non-essential amino acids, penicillin G (100 IU/ml) and streptomycin (100 μg/ml)]. Cells were then stimulated with PPD antigen (1·0 μg/ml), phytohaemagglutinin (PHA, 10 μg/ml; Sigma) or lipopolysaccharide (LPS, 0·1 μg/ml; Sigma), and incubated at 37°C in a 5% CO_2 humidified air atmosphere until used for either RNA isolation or supernatant fluid collection.

Flow cytometry

Cell suspensions were stained for analysis by flow cytometry in V-bottomed tubes (3×10^6 cells per test). Flow cytometry data were collected using a FACSCalibur machine (Becton Dickinson, Oxnard, CA, USA) and analysed with CELL QUEST software (Becton Dickinson).

Lymphocyte proliferation assay

PBMC ($2·5 \times 10^4$ per well) were placed in each well of a round-bottomed microtitre tissue culture plate (Falcon Products, Becton Dickinson). The blastogenic response was measured at various PPD antigen concentrations for 5 days at 37°C in a 5% CO_2 humidified air atmosphere. Based on dose–response studies, the optimum concentration of PPD antigen in the final culture was 1·0 μg/ml (data not shown). PHA was used at a concentration of 10 μg/ml as a positive control for cell reactivity. Cells were incubated for 5 days at 37°C in a 5% CO_2 humidified air atmosphere. PHA-stimulated cultures were incubated for 3 days, and 2 μCi[^{3}H] of thymidine (Amersham, UK) were added for the final 18 h. Cells were harvested on fibreglass paper using a cell harvester (Cambridge Technology, Watertown, MA, USA), and the incorporated radioactivity was measured in a liquid scintillation counter (Beckman, Somerset, NJ, USA). The results were expressed as the mean counts per min ± the standard error (s.e.) of triplicate cultures for each donor. The stimulation index (SI) was calculated using this value and the counts per mine obtained in unstimulated cultures.

Enzyme-linked immunosorbent assay for IFN-γ, IL-12, IL-18 and IL-10

Supernatant fluids were collected from cultures of PBMC stimulated with PPD antigen at 16 (for IL-12 and IL-18), 48 (for IL-10) and 96 (for IFN-γ) h, and were then frozen at –80°C. The frozen supernatant fluids were thawed at room temperature, and cytokine levels were measured with commercial assay kits for IFN-γ, IL-12 p70, IL-12 p40, IL-10 (PharMingen) and IL-18 (R & D Systems), according to the manufacturers' instructions. Cytokine concentrations in the samples were calculated with standard curves generated from recombinant cytokines, and the results were expressed in picograms per millilitre. The difference between duplicate wells was consistently less than 100% of the mean.

Statistical methods

The results are presented as the mean ± s.d. Statistical significance was calculated using either Student's *t*-test or linear regression analysis.

RESULTS

CD4-positive T-cell counts, PPD-stimulated lymphoproliferative responses and IFN-γ production in patients with MDRTB compared with HTR

CD4+ T cell counts and lymphoproliferative responses. As shown in Fig. 1(a), the $CD4^+$ T-cell counts of MDRTB patients were significantly lower than those of HTR (mean 1409·9 ± 1038·1 *versus* 2044·9 ± 695·3 μl^{-1}, $P < 0·05$). In addition, the lymphoproliferative responses to PPD antigen were significantly lower in PPD-stimulated PBMC from MDRTB patients than in cells from HTR (mean 4118·1 ± 4658·8 *versus* 18 601·7 ± 8315·5, $P < 0·001$; Fig. 1b). A majority of the MDRTB patients did not recognize (SI < 4·0) either of the antigens (12 of 18 [64·7%]). All of the non-reactors showed either no stimulation ($n = 7$; SI < 2·0), or only a marginal increase ($n = 5$; SI = 2·0–4·0) in the lymphocyte response to PPD. Furthermore, three MDRTB patients with $CD4^+$ T-cell counts below 500 μl^{-1} had no stimulation (SI < 2·0) of lymphoproliferative responses to PPD.

In HTR, the background CPM incorporated was 920·1 ± 350·5, and positive responses ranged from 3459·0 to 38 986·0. To assess the proliferative response under maximal conditions, PBMC were stimulated with the polyclonal mitogen PHA. Stimulation of PBMC with PHA resulted in greater lymphoproliferation (50 000–200 000) than PPD in both groups tested (data not shown).

IFN-γ. Individual data on IFN-γ production by PBMC were obtained following 96 h stimulation with PPD (Fig. 1c). The mean IFN-γ concentrations of MDRTB patients were significantly lower than corresponding values in HTR (mean 403·1 ± 352·1 *versus* 1877·2 ± 745·6 pg/ml, $P < 0·001$). Production of IFN-γ in all patients was significantly correlated with $CD4^+$ T-cell counts ($n = 18$, $r = 0·85$, $P < 0·001$; Fig. 1d). Furthermore, PPD-induced IFN-γ production was significantly correlated with lymphoproliferative responses in MDRTB patients ($n = 18$, $r = 0·68$, $P < 0·01$; Fig. 1d).

Stimulation of PBMC with PHA resulted in the secretion of IFN-γ (3000–15 000 pg/ml), and the mean IFN-γ production in response to PHA was similar to HTR (data not shown), indicating that there is no absolute qualitative defect in IFN-γ production in these patients.

IL-18 and IL-10 production after in vitro *PPD stimulation in patients with MDRTB compared with HTR*

IL-18. PBMC from MDRTB and HTR produced comparable concentrations of IL-18, as determined by ELISA. As shown in Fig. 2(a), the mean concentration of IL-18 in PBMC from MDRTB patients was significantly increased after an 18 h PPD

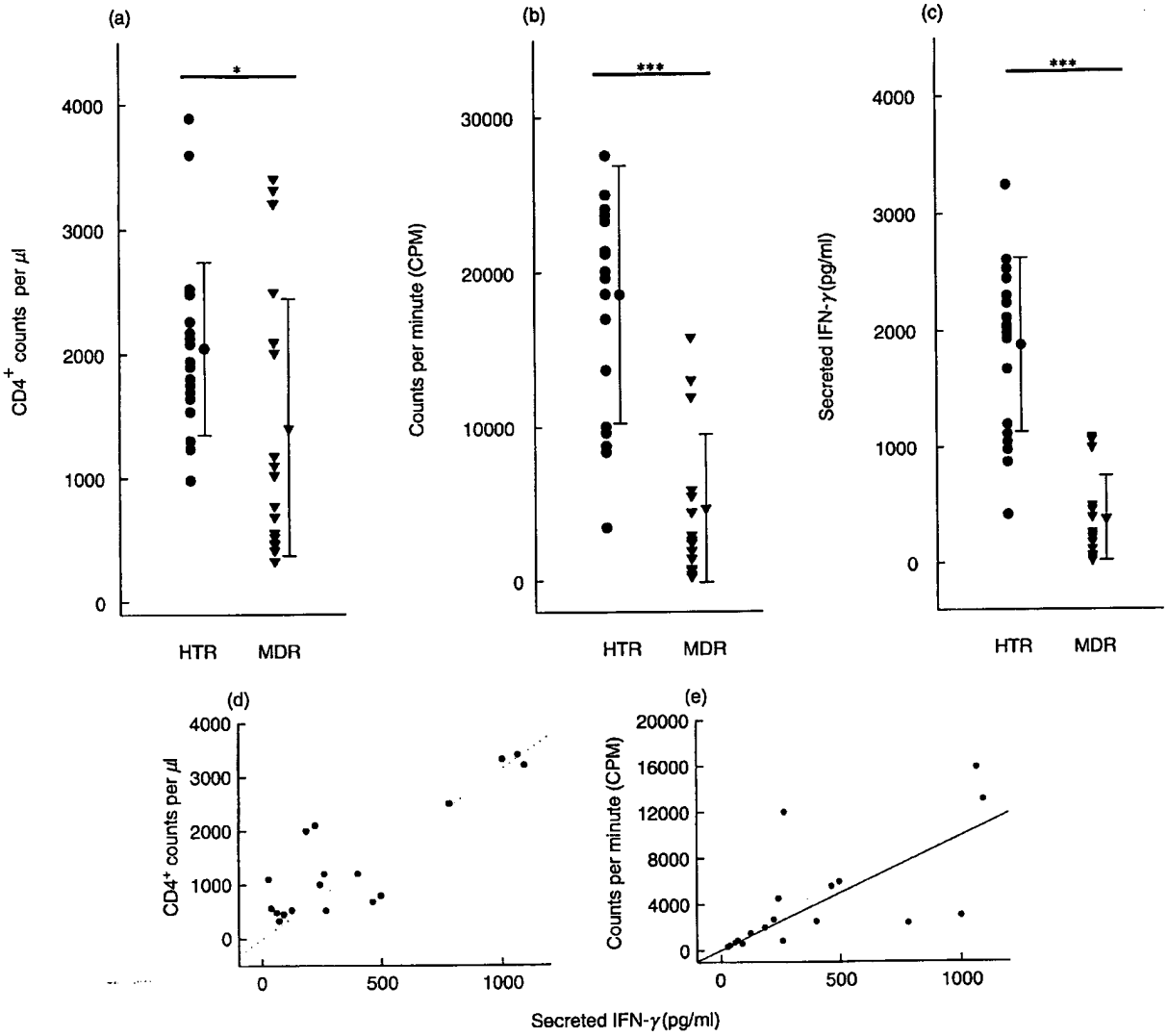

Fig. 1. Lymphoproliferative responses, CD4 counts and IFN-γ production in PBMC from patients with MDRTB and HTR in response to the PPD antigen of *Mycobacterium tuberculosis*. (a) PBMC were isolated and FACS analysis performed. CD4 count was calculated based on a concomitant complete blood count. (b) PBMC were stimulated for 5 days with the PPD antigen of *M. tuberculosis* at a concentration of 1·0 μg/ml. Proliferative responses were assessed as [^{3}H]-thymidine incorporation in PBMC from healthy controls and TB patients. Incorporation of [^{3}H]-thymidine occurred during the last 18 h of a 5 day culture; unstimulated PBMC served as controls. (c) PBMC were stimulated for 96 h with PPD antigen at a concentration of 1·0 μg/ml. Supernatant fluids were prepared following a 96 h stimulation with PPD, and IFN-γ production was measured using ELISA. Values are the mean ± s.d. of triplicate supernatant samples. *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$ (Student's *t*-test). Significant correlations were found between (d) IFN-γ and CD4 counts ($n = 18$, $r = 0·85$, $P < 0·001$), and (e) between IFN-γ and lymphoproliferative responses in TB patients ($n = 18$, $r = 0·68$, $P < 0·01$).

stimulation compared with those from HTR (mean 405·4 ± 252·6 *versus* 159·9 ± 63·1 pg/ml, $P < 0·01$) (Fig. 2a). We found that unstimulated PBMC also produced comparative values of IL-18 (121·8 ± 66·7 pg/ml). Therefore, IL-18 production in HTR was not shown to be up-regulated following 18 h of PPD stimulation compared with other stimuli, such as adherence alone. An insignificant correlation between IL-18 production and IFN-γ production was observed in culture supernatant fluids from MDRTB patients ($n = 18$, $r = -0·06$, $P > 0·05$; Fig. 2c). Stimulation of PBMC with LPS resulted in the secretion of IL-18 (1000–2000 pg/ml). LPS induced similar IL-18 titres in the healthy controls and TB patients (data not shown).

IL-10. Although there was a substantial heterogeneity in the production of IL-10 in PBMC from MDRTB patients, PBMC from MDRTB patients produced significantly more IL-10 after stimulation with PPD, compared with HTR (mean 588·1 ± 509·2 *versus* 305·4 ± 244·7 pg/ml, $P < 0·05$) (Fig. 2b). An insignificant correlation was observed between IL-18 and IFN-γ production in culture supernatant fluids from MDRTB patients ($n = 18$, $r = 0·34$, $P > 0·05$; Fig. 2d). Stimulation of PBMC with LPS preferentially

induced the secretion of IL-10 (500–1000 pg/ml). LPS induced similar IL-10 titres in HTR and patients (data not shown).

IL-12 production after in vitro *stimulation with PPD in patients with MDRTB*

We were interested in determining whether MDRTB patients had a lower endogenous IL-12 production that was correlated with their deficient IFN-γ response. As shown in Fig. 3(a), there was no statistically significant difference between the MDRTB patients and the HTR group, although the mean IL-12 p40 production was higher in MDRTB than in HTR (mean 1886·9 ± 2123·2 *versus* 603·3 ± 511·7 pg/ml, $P > 0.05$).

In addition, we measured the release of IL-12 p70, which is a more reliable indicator of biologically active IL-12 production (Fig. 3b). Although excess IL-12 p70 protein (from 20·0 to 110·0 pg/ml) was detected in some donors with IL-12 p40 protein levels exceeding 1000 pg/ml, the PPD-induced IL-12 p70 levels were very low. The PPD-stimulated PBMC from MDRTB patients did not show any significant difference compared with those from HTR (mean 19·4 ± 31·4 *versus* 4·7 ± 6·3 pg/ml, $P > 0.05$).

In addition, IL-12 p70 release was significantly correlated with the release of IL-12 p40 ($n = 15$, $r = 0.83$, $P < 0.001$; data not shown). Unstimulated PBMC showed no detectable levels of either IL-12 p40 (<100·0 pg/ml) or IL-12 p70 (<0·2 pg/ml, data not shown). Stimulation of PBMC with LPS resulted in the secretion of IL-12 p40 (1200–2500 pg/ml). LPS induced similar IL-12 titres in MDRTB and HTR (data not shown).

Although we did not observe a significant correlation between IL-12 p40 and IFN-γ production in culture supernatant fluids from MDRTB patients ($n = 18$, $r = 0.24$, $P > 0.05$; Fig. 3d), there was a significant correlation between IL-12 p40 and IL-10 production ($n = 18$, $r = 0.94$, $P < 0.001$; Fig. 3c). This relationship was also observed in HTR ($n = 9$, $r = 0.75$, $P < 0.001$; data not shown).

Some MDRTB patients exhibited IL-12 p40 production more than two s.d. above the mean IL-12 p40 concentration in HTR (>1626·8 pg/ml; $n = 4$; mean 3406·3 ± 2030·6 pg/ml). Although there were no significant differences between these patients and others (age, nutrition, chest X-ray findings, IFN-γ levels and CD4 counts), these patients had a history of infection of less than 4 years. Patients with lower IL-12 had a longer history of infection (>10 years; $n = 4$).

Effect of neutralization of endogenous IL-10 on PPD-induced IL-12 p40 and IFN-γ production

Next, we assessed the effect of endogenous IL-10 neutralization on PPD-induced IL-12 p40 and IFN-γ production. PBMC from MDRTB patients were cultured in complete RPMI, with or without PPD (1 μg/ml), in the presence or absence of neutralizing antibodies to IL-10 (2 μg/ml). In preliminary experiments, the amounts of neutralizing antibodies were found to abrogate the bioactivity of 300 pg of IL-10 per ml. Culture supernatant fluids were collected at 18 and 96 h and assayed for IL-12 p40 and IFN-γ immunoreactivity. PPD-induced IL-12 p40 levels doubled in cultures containing neutralizing antibody to IL-10 in HTR and MDRTB patients, as shown in Fig. 4 ($P < 0.001$, HTR;

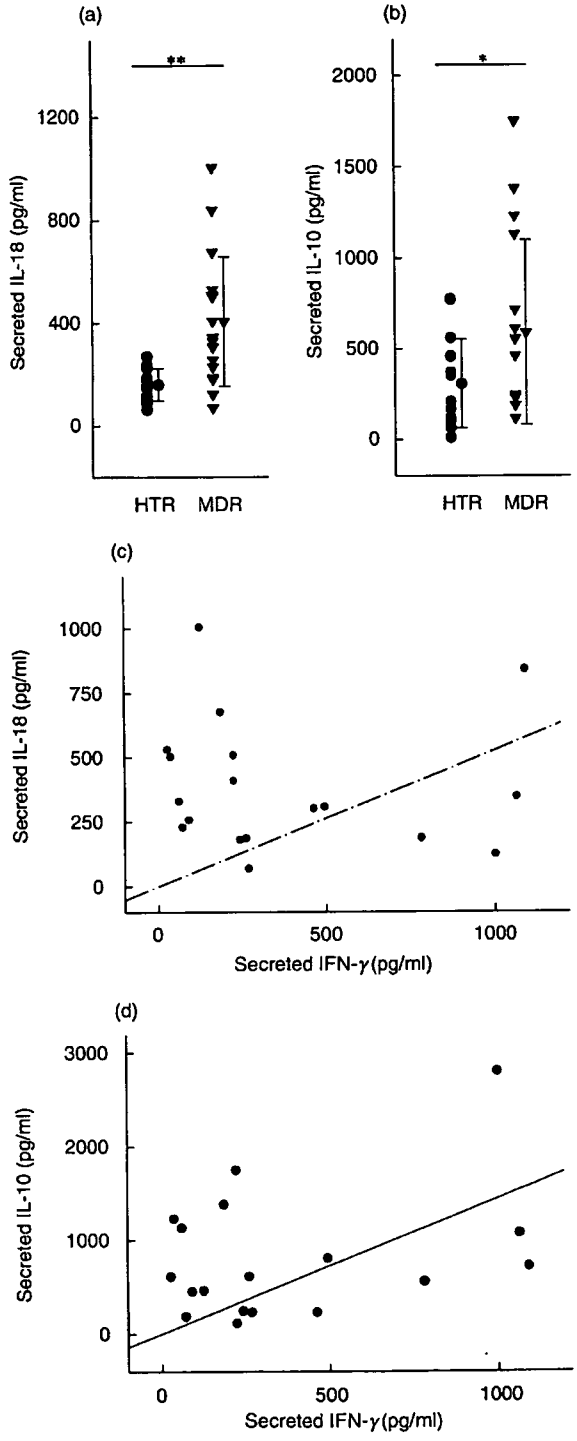

Fig. 2. IL-18 and IL-10 production in PBMC from patients with MDRTB in response to the PPD antigen of *M. tuberculosis*. (a) IL-18 and (b) IL-10 production in PBMC were determined after *in vitro* stimulation with PPD. Supernatant fluids were prepared following a 96 h stimulation with PPD, and cytokine production was measured using ELISA. Values are the mean ± s.d. of triplicate supernatant samples. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's *t*-test). No significant correlations were found (c) between IFN-γ and IL-18 ($n = 18$, $r = -0.06$, $P > 0.05$), or (d) between IFN-γ and IL-10 production in TB patients ($n = 18$, $r = 0.34$, $P > 0.05$).

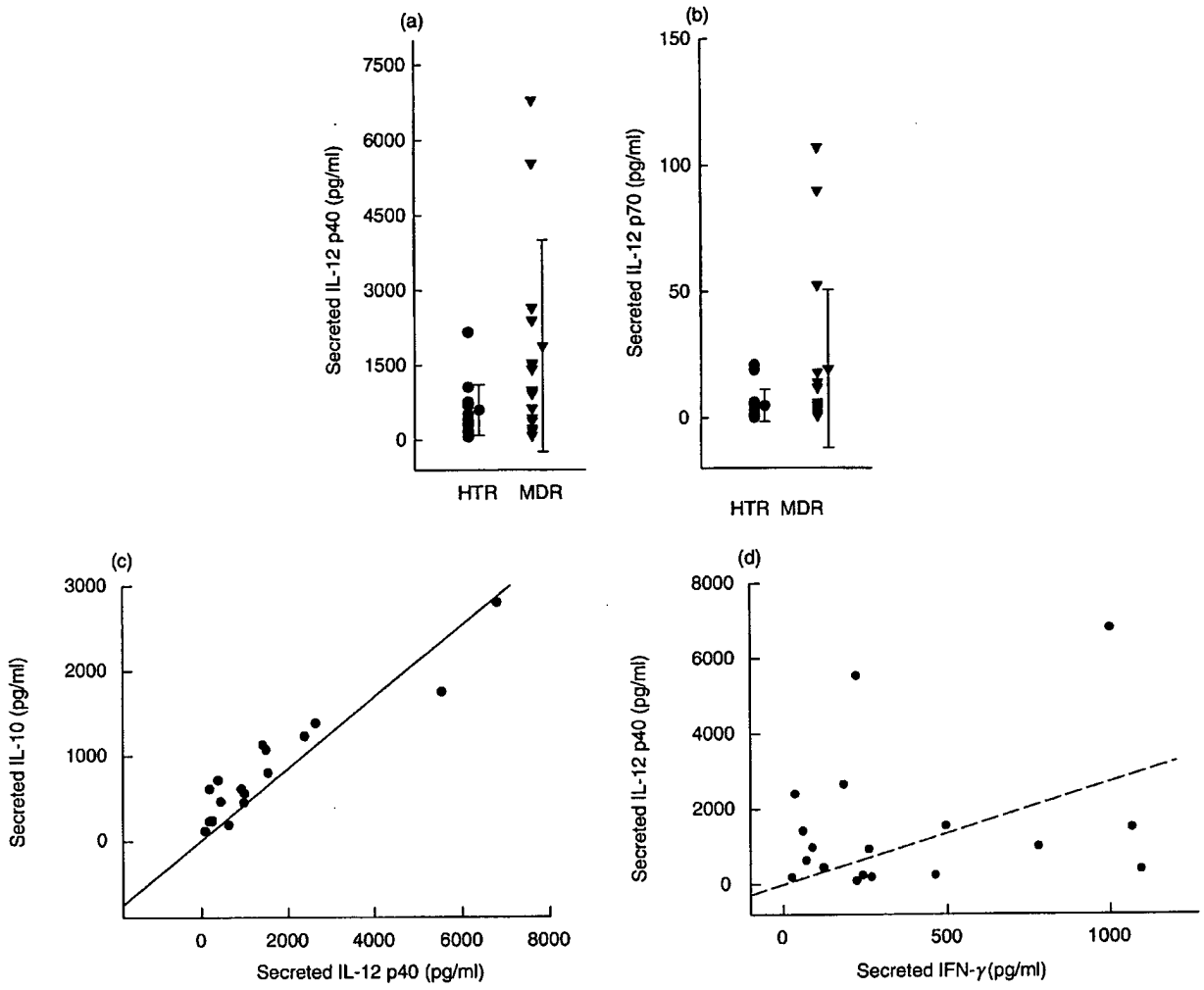

Fig. 3. IL-12 production in PBMC from patients with MDRTB in response to the PPD antigen of *M. tuberculosis*. IL-12 p40 and p70 production in PBMC were determined after *in vitro* stimulation with PPD antigen. Supernatant fluids were prepared after 18 h and cytokine concentrations were measured using ELISA. Values are the mean ± s.d. of triplicate supernatant samples. (a) IL-12 p40 production in MDRTB patients. (b) IL-12 p70 production in MDRTB patients. A significant correlation was found between (c) IL-12 p40 and IL-10 ($n = 18$, $r = 0{\cdot}94$, $P < 0{\cdot}001$), whereas no significant correlation was found between (d) IFN-γ and IL-12 p40 production in TB patients ($n = 18$, $r = 0{\cdot}24$, $P > 0{\cdot}05$).

$P < 0{\cdot}01$, MDRTB). In addition, co-culture with neutralizing antibody to IL-10 led to a significant increase in IFN-γ production in HTR (1·6-fold, $P < 0{\cdot}05$; Fig. 4). However, there was no significant increase in IFN-γ production after co-culture with neutralizing antibody to IL-10 in MDRTB patients (1·2-fold, $P > 0{\cdot}05$; Fig. 4).

DISCUSSION

Our previous work and several other studies showed that PBMC proliferation and IFN-γ production in PBMC were reduced in patients with active TB and advanced disease [24–27]. In addition, *M. tuberculosis*-stimulated IFN-γ production by patients with pulmonary TB was found to deteriorate as their disease worsened [27,28]. Our study found that HIV-negative MDRTB patients, who had been infected for at least 2 years, also exhibited depressed IFN-γ production after mycobacterial antigen stimulation. Our findings support earlier indications that HIV-negative MDRTB patients with CD4 counts below 500 per μl have markedly decreased lymphoproliferation and IFN-γ production in response to PPD or *M. tuberculosis* [9]. One possibility is that the diminished IFN-γ responses in MDRTB patients are due to the loss of *M. tuberculosis*-reactive T cells through apoptotic mecha-

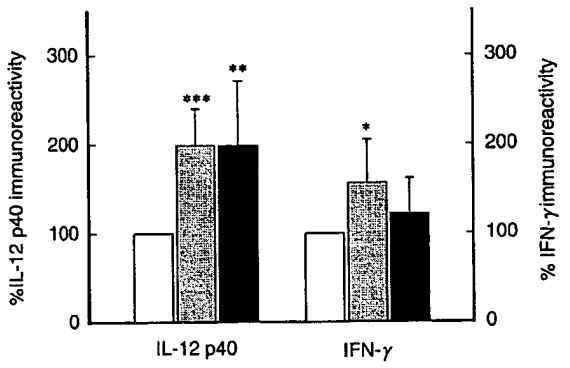

Fig. 4. Effect of endogenous IL-10 on PPD-induced IL-12 p40 and IFN-γ production by PBMC. PBMC from HTR (n = 10) and MDRTB patients (n = 9) were cultured with or without neutralizing antibody to IL-10 (2 μl). PPD (1 μg/ml) was added to all cultures. The immunoreactivity for IL-12 p40 and IFN-γ was assessed in culture supernatant fluids at 18 and 96 h, respectively. The percent increase in IFN-γ and IL-12 p40 immunoreactivity compared with those of PPD alone (100%) is shown. (□) PPD only; (▨) a-IL-10 (HTR); (■) a-IL-10 (MDRTB).

nisms [29]. A previous study of *M. tuberculosis*-induced apoptosis in active pulmonary TB patients showed a reduction in the rate of cell apoptosis and increased IFN-γ production after successful anti-tuberculosis chemotherapy [29]. Thus, apoptotic pathways may contribute to T-cell hyporesponsiveness through prolonged deletion of mycobacterial antigen-sensitive T cells in these chronic TB patients with treatment failure.

Protective immunity against *M. tuberculosis* requires both activated mononuclear phagocytes and T cells. IL-12 may provide a crucial link between these two cell populations by regulating IFN-γ production and the cytotoxic effector function of mycobacterial antigen-specific T cells [30]. Our previous data demonstrated that depressed IL-12 and IFN-γ production recovered in active pulmonary TB patients after anti-tuberculosis therapy [24]. We investigated whether IL-12 depression is associated with the reduced IFN-γ production seen in MDRTB patients. However, the patients in this study showed extremely variable patterns of IL-12 production, and no significant correlation was observed between IFN-γ and IL-12. Although we could not observe any overall correlation between IL-12 and various clinical factors (age, nutrition, chest X-ray findings, IFN-γ levels and CD4 counts), the patient group with higher IL-12 levels (p40, >1630 pg/ml; n = 4) had a shorter history of infection (<4 years), whereas others with lower IL-12 levels (p40, <300 pg/ml; n = 4) had a longer history (>10 years).

A recent study of TB patients with differing pulmonary involvement revealed that IL-12 synthesis was only augmented in advanced TB cases, who also displayed lower IFN-γ production, when compared with moderate cases and HTR [28]. Since all the patients in our study were classified as very advanced disease as assessed by X-ray, the results indicate that patients with advanced disease have augmented IL-12 levels. In addition, our data increase the knowledge of IL-12 depression in chronic TB patients with long-standing infection. Long-term follow-up of the patients will clarify the picture of IL-12 secretion and its role in the pathogenesis of chronic progressive TB.

The heterogenous IL-12 profile seen in MDRTB patients does not appear to be directly involved in the individual variation in IL-12 producing cells. The number of monocyte-derived macrophages, the main source of IL-12, was normal in most of these patients, and they showed similar IL-12 production in response to LPS when compared with HTR (data not shown). However, we could not examine whether monocyte-derived or CD83(+) blood dendritic cells were normal. Further experiments on dendritic cells in MDRTB patients should clearly demonstrate whether the very depressed IL-12 secretion in some patients is mediated by depressed dendritic cell function.

Our findings of augmented IL-12 levels in MDRTB patients with disease for less than 3 years may be due to restored IFN-γ production. A previous report suggests that reduced IFN-γ production by PBMC in TB patients is correlated with poor IL-12Rβ1 and IL-12Rβ2 expression [31]. In addition, there are reports of the significance of IL-12Rβ2 in leprosy patients. IL-12Rβ2 was more strongly expressed in lesions from tuberculoid patients (the resistant form of leprosy) than in those from lepromatous patients (the susceptible form of leprosy), whose cells do not respond to IL-12 [32]. Previous studies and our data led us to hypothesize that depressed IL-12R production may lead to IL-12 hyporesponsiveness in the $CD4^+$ T cells of these patients.

Since IL-12 production was significantly correlated with IL-10 levels, we examined whether a concomitant increase in IL-10 plays a role in the down-regulation of IFN-γ production. Interestingly, IFN-γ was not significantly increased in MDRTB patients, in spite of IL-12 p40 increases after endogenous IL-10 neutralization. Several studies have reported increased IL-10 production in active pulmonary TB patients [24,26]. Recent data showed that TGF-β1 and IL-10 together potentiate the modulatory effect on *M. tuberculosis*-induced T-cell production of IFN-γ, and TGF-β1 alone enhances IL-10 production. At sites of active *M. tuberculosis* infection, these interactions might be conducive to the suppression of mononuclear cell function [33]. We did not test the effect of adding anti-TGFβ1 or anti-IL-4 to cultures to determine whether IFN-γ or IL-12 was increased in MDRTB patients. Studies in TGF-β1 indicate that expression of both IL-12 p40 and p35 is suppressed by TGF-β1, and that TGF-β1 interferes with the bioactivity of IL-12 in enhancing *M. tuberculosis*-induced IFN-γ production [34]. Thus, we cannot rule out a TGF-β1 effect on IFN-γ reduction in MDRTB patients.

Earlier studies in patients with MDRTB have revealed impaired Th1 responses; however, little is known about IL-18 production. We found that both IL-18 and IL-10 production were significantly elevated in MDRTB after stimulation with PPD, and these elevations were not correlated with IFN-γ production. IL-18 is a pro-inflammatory cytokine, initially isolated from liver cells [17,35], that has a pleiotropic function which participates in the induction of IFN-γ and other cytokines [11]. In animals, IL-18 contributes to protective immunity against a variety of pathogens, including *Cryptococcus*, *Leishmania*, *Salmonella* and *M. tuberculosis* [20,36–39]. In humans, the role of IL-18 is controversial. IL-18 production in response to mycobacterial antigens correlates strongly with IFN-γ production and with protective immunity to mycobacteria [40,41]. However, PBMC from active pulmonary TB patients showed significantly enhanced IL-18 proteins after 96 h of stimulation [24], suggesting a pro-inflammatory role in TB. Furthermore, circulating IL-18 correlated with the extent of disease in pulmonary TB; it was significantly higher in far advanced pulmonary TB than in patients with minimal TB

or HTR [42]. We found that MDRTB patients with advanced disease have increased levels of IL-18, suggesting its role in immunopathogenesis.

In conclusion, these data demonstrate that MDRTB patients showed significantly decreased IFN-γ production compared with HTR, although their IL-18 and IL-10 levels were higher after stimulation with PPD antigen. Furthermore, the IL-12 response to PPD was correlated with IL-10 production, but not with IFN-γ production. These results suggest that production of IL-12, IL-18 and IL-10 may be dysregulated in MDRTB patients, and further characterization of the cytokine modulation may provide clarification of the possible application of adjunctive immunotherapy to the treatment of MDRTB.

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Evaluation of the effect of interleukin-10 on the multiplication of Mycobacterium avium complex in human macrophages and in C57BL/6 mice.

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OBJECTIVE: To examine the effect of recombinant human IL-10 (rhIL-10) on MAC infection of human macrophages and C57BL/6 mice. METHODS: We compared rhIL-10 with the effects of the immunosuppressive drugs prednisolone and cyclosporin A, both in vitro and in vivo. RESULTS: There was no effect of rhIL-10 on the multiplication of MAC in human macrophages after 1 week of infection. In C57BL/6 mice, rhIL-10 at 2.5 or 25 m g/mouse had no additional multiplicatory effect after 3 weeks of infection, while the spleens of mice treated with prednisolone had 600% higher bacteria than controls or rhIL-10-treated mice (p0.01). CONCLUSIONS: These data suggest that rhIL-10 does not further decrease the resistance of human macrophages and C57BL/6 mice to MAC infection, whereas prednisolone leads to increased multiplication of MAC in the spleens of infected C57BL/6 mice. These results may be of interest in the context of the therapeutic use of rhIL-10 in some autoimmune disorders.

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In Vivo IL-10 Production Reactivates Chronic Pulmonary Tuberculosis in C57BL/6 Mice[1]

Joanne Turner,[2]* Mercedes Gonzalez-Juarrero,* Debi L. Ellis,* Randy J. Basaraba,* Andre Kipnis,* Ian M. Orme,* and Andrea M. Cooper†

The production of immunosuppressive cytokines, such as IL-10 and TGF-β, has been documented in individuals diagnosed with active tuberculosis. In addition, IL-10 production is increased within the lungs of mice that have chronic mycobacterial infection. Therefore, we hypothesized that the down-regulatory properties of IL-10 might contribute to the reactivation of chronic *Mycobacterium tuberculosis* infection in mice. To determine the influence of IL-10 on the course of infection, transgenic mice producing increased amounts of IL-10 under the control of the IL-2 promotor were infected with *M. tuberculosis* via the respiratory route. Mice that overexpressed IL-10 showed no increase in susceptibility during the early stages of infection, but during the chronic phase of the infection showed evidence of reactivation tuberculosis with a highly significant increase in bacterial numbers within the lungs. Reactivation was associated with the formation of macrophage-dominated lesions, decreased mRNA production for TNF and IL-12p40, and a decrease in Ag-specific IFN-γ secretion. These data support the hypothesis that IL-10 plays a pivotal role during the chronic/latent stage of pulmonary tuberculosis, with increased production playing a potentially central role in promoting reactivation tuberculosis. *The Journal of Immunology*, 2002, 169: 6343–6351.

It is estimated that one-third of the world's population harbors an infection with *Mycobacterium tuberculosis*, of which ~3 million individuals go on to develop active disease each year (1). The factors that contribute to an individual's susceptibility to reactivate an apparently latent *M. tuberculosis* infection are currently unknown, although malnutrition and immunosupression are among some of the suggested reasons (2, 3). A current area of active research is that of determining genetic linkage for susceptibility to develop reactivation tuberculosis. In man, several genes such as *Nramp1* (4, 5), *15q*, and *Xq* (6) have been linked to tuberculosis suggesting that susceptibility cannot be conferred by a single gene alone but is in fact multifactoral in nature. This theory is supported by studies in the mouse model that have identified associations with loci on chromosomes 3 and 9 (7), the H-2 locus (8), and more recently the *sst1* gene (9), suggesting that susceptibility is very unlikely to be mapped to a single gene. Therefore, the identification of immune correlates of protection may be a more feasible approach with which to identify the very large number of latently infected individuals who are at risk of developing reactivation disease.

Using mouse models of reactivation tuberculosis, we have previously demonstrated that mouse strains such as the CBA/J and DBA/2 fail to up-regulate the cell surface adhesion molecules CD11a and CD54 on their circulating lymphocytes during an infection with *M. tuberculosis* (10). The failure of circulating T cells to increase their expression of adhesion molecules correlated with the absence of lymphocyte foci within the lung granulomas. We hypothesized that this absence of lymphocytes contributed to the eventual breakdown of the lesion and reactivation of tuberculosis, and that the measurement of CD11a and CD54 expression on circulating lymphocytes might be a useful correlate of protection. It is likely that the failure to up-regulate the expression of these molecules is the consequence of an upstream event, although the mechanisms contributing to this event in the reactivation susceptible mouse strains are currently unknown.

In this regard, IL-10 is a cytokine that has been shown to have immunosuppressive activity that may contribute to mycobacterial disease. In the presence of IL-10, it has been shown that both T cell proliferation and IFN-γ production is inhibited (11–14) and the action of IL-10 has been linked to its down-regulation of macrophage activation. IL-10 can inhibit TNF and NO secretion (15, 16), down-regulate the expression of costimulatory molecules (11), and MHC class II (16, 17); therefore, it compromises both macrophage microbicidal mechanisms and Ag presentation. The ability of IL-10 to down-regulate immune responses and the fact that IL-10 can be detected in tuberculosis patients (18, 19) have led researchers to investigate whether IL-10 plays a role in susceptibility to tuberculosis. More specifically, it has been suggested that the removal of IL-10 will enhance protective immunity. Despite the known immunosuppressive properties of IL-10, infection of IL-10 gene-disrupted mice with *Mycobacterium bovis* bacillus Calmette-Guérin resulted in only a small and transient increase in resistance to infection, and the outcome of chronic disease was unaltered (20, 21). More significantly, infection of IL-10 gene-disrupted mice with *M. tuberculosis* resulted in either a minor increase in resistance (22) or no increase at all (23). The studies described used gene-disrupted mice that were bred onto a naturally resistant mouse strain, capable of generating a vigorous immune response during *M. tuberculosis* infection and showing no signs of reactivation disease until well into old age (24, 25). In this regard, we hypothesized that if C57BL/6 IL-10 gene-disrupted mice were slightly more resistant to tuberculosis, the overexpression of IL-10

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may result in an increased susceptibility of this mouse strain to infection perhaps more akin to the susceptible mouse strains that we have previously characterized.

To determine whether increased IL-10 production can influence the outcome of disease, we chose to study *M. tuberculosis* infection in transgenic C57BL/6 mice that express IL-10 under the control of the IL-2 promoter (IL-10 transgenic mice) (26). These mice thus generate excess IL-10 when the IL-2 gene is induced, such as occurs during the vigorous response seen in *M. tuberculosis*-infected C57BL/6 mice. Our data demonstrate that the increased production of IL-10 during an infection with *M. tuberculosis* conferred a reactivation susceptible phenotype on the naturally resistant C57BL/6 mouse strain. Lesions within the lungs of IL-10 transgenic mice were dominated by macrophages, mRNA for TNF and IL-12p40 was reduced in the lung, and the capacity of lung cells to secrete Ag-specific IFN-γ was compromised. This phenotype closely resembles that seen in the reactivation susceptible mouse strains. The demonstration that lesions within the lungs of CBA/J mice infected with *M. tuberculosis* contain substantial levels of IL-10 supports the hypothesis that this cytokine plays a major role in the development of reactivation tuberculosis in man.

Materials and Methods

Mice

These studies were performed using specific pathogen-free C57BL/6 and CBA/J mice (The Jackson Laboratory, Bar Harbor, ME) at 6–8 wk of age. The IL-10 transgenic mice were a kind gift from Drs. H. Cheroute and M. Kronenberg (La Jolla Institute for Allergy and Infectious Disease, La Jolla, CA) (26). Mice were kept in ABL-3 biohazard conditions throughout the study and maintained on sterile chow and water ad libitum. The specific pathogen-free nature of the mouse colonies was demonstrated by testing sentinel animals. These were shown to be negative for 12 known mouse pathogens. All experimental protocols were approved by the Colorado State University Animal Care and Users Committee.

Bacterial infections

M. tuberculosis strain Erdman was grown from low passage seed lots in Proskauer-Beck liquid media containing 0.02% Tween 80 to mid-log phase, then aliquoted and frozen at −70°C until use. Mice were infected via the aerosol route with a low dose (10^2) of bacteria. Briefly, the nebulizer compartment of a Middlebrook airborne infection apparatus (Glas-col, Terre Haute, IN) was filled with 5 ml of distilled water containing a suspension of bacteria known to deliver ~100 bacteria/lung. The numbers of viable bacteria in the lungs were followed against time by plating serial dilutions of individual partial organ homogenates onto nutrient Middlebrook 7H11 agar and counting bacterial colony formation after 21 days incubation at 37°C. The data were expressed as the $\log_{10}$ value of the mean number of bacteria recovered from four individual animals.

Histology

The right caudal lung lobe from each mouse (n = 4/group) was infused with 10% neutral-buffered formalin. Tissues were sectioned for light microscopy with lobe orientation designed to allow for the maximum surface area of each lobe to be seen. Sections were stained with H&E. Sections were examined by a veterinary pathologist without prior knowledge of the experimental groups, and evaluated at least twice to verify the reproducibility of the observations.

Immunohistochemistry

Formalin-fixed lung tissue was embedded in paraffin, and serial sections of tissue were cut 7-μm thick. Paraffin was removed using EZ-DeWax solution (BioGenex Laboratories, San Ramon, CA), and the Ag retrieval procedure was performed using the Ag Retrieval Citra solution according to the manufacturer's protocol (BioGenex Laboratories). Tissue endogenous peroxidase was inactivated using peroxidase block reagent (Innogenex, San Ramon, CA) and nonspecific binding blocked by incubating the sections for 30 min with 5% mouse serum (Sigma-Aldrich, St. Louis, MO). The sections were incubated overnight at 4°C with goat polyclonal Ab specific for murine IL-10, or an irrelevant IgG goat Ab (Santa Cruz Biotechnology, Santa Cruz, CA). The sections were washed with PBS containing 0.5% Tween 20 followed by incubation with the secondary polyclonal donkey anti-goat Ab conjugated to HRP (Serotec, Raleigh, NC) for 40 min at room temperature. Samples were developed using AEC substrate (BioGenex Laboratories). Sections were counterstained using hematoxylin (BioGenex Laboratories) and mounted using crystal mount.

Isolation of cells from infected lungs and spleen

Mice were euthanized and the pulmonary cavity opened. The lung was cleared of blood by perfusing through the pulmonary artery with 10 ml of saline containing 50 U/ml of heparin (Sigma-Aldrich). Lungs were removed from the pulmonary cavity and placed in cold DMEM (Life Technologies, Grand Island, NY). After removal of the connective tissue and trachea, the lungs were disrupted using sterile razor blades and incubated for 30 min at 37°C in a final volume of 2 ml DMEM containing collagenase XI (0.7 mg/ml; Sigma-Aldrich) and type IV bovine pancreatic DNase (30 μg/ml; Sigma-Aldrich). Ten milliliters of DMEM containing supplements (10% heat-inactivated FCS; Life Technologies), 1% 1 M HEPES buffer (1 M; Sigma-Aldrich), 1% L-glutamine (200 nM; Sigma-Aldrich), and 2% MEM-nonessential amino acids (100×; Sigma-Aldrich) was added to stop the action of the enzyme. Digested lungs were then gently dispersed through a nylon screen, and centrifuged at 300 × g. Remaining RBCs were lysed using ACK lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$). Cells were resuspended in DMEM plus supplements. Spleens were harvested from individual mice and the cells dispersed through a nylon screen. RBCs were lysed using ACK lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$) and spleen cells were resuspended in DMEM plus supplements.

Flow cytometry

Cells from the lung or spleen were obtained from each individual mouse and incubated with specific Ab (25 μg/ml) for 30 min at 4°C and in the dark followed by two washes in D-RPMI lacking biotin and phenol red (Irvine Scientific, Santa Ana, CA). Cells were analyzed using a BD Biosciences FACSCalibur and data analyzed using CellQuest (BD Biosciences, San Diego, CA). Lymphocytes were gated by forward and side scatter, and $CD4^+$ and $CD8^+$ T cells identified by the presence of specific fluorescent-labeled Ab. Cell surface markers were analyzed using FITC-labeled anti-CD11a (clone 2D7) and PE anti-CD54 (clone 3E2), PerCP-labeled anti-CD4 (clone RM4-5), and allophycocyanin-labeled anti-CD8 (clone 53-6.7). Appropriate isotype control Abs were included in each analysis. All Abs were purchased from BD PharMingen (San Diego, CA). Measurement of intracellular IFN-γ was conducted by preincubating lung cells with 0.1 μg/ml anti-CD3ε (clone 145-2C11) and 1 μg/ml anti-CD28 (clone 37.51) in the presence of 3 μM monensin for 4 h at 37°C, 5% CO_2. Cells were stained with Abs for cell surface molecules as described above before a permeabilization step conducted according to the manufacturer's instructions (Fix/Perm kit; BD PharMingen). FITC anti-IFN-γ (clone XMG1.2) or IgG1 isotype control Ab was incubated with the cells for a further 30 min, washed twice, and resuspended in D-RPMI before analysis.

Lung cell culture

Lung cells were resuspended at 5 × 10^6 cells/ml in DMEM plus supplements. Cells were cultured with OVA (10 μg/ml; Sigma-Aldrich), or culture filtrate proteins (CFPs)[3] from *M. tuberculosis* (10 μg/ml; received from National Institutes of Health contract AI-75320). After 5 days of culture at 37°C and 5% CO_2, the plates were frozen at −70°C until further analysis.

Cytokine ELISA

Supernatants were harvested from lung cell cultures and assayed for the presence of IFN-γ and IL-10 by ELISA. Briefly, the primary Ab (IFN-γ clone R4-6A2, IL-10 clone JES5.2A5; BD PharMingen) was incubated overnight in 96-well round bottom Immulon 2 plates in carbonated coating buffer. Excess Ab was washed away using PBS-Tween 20. The wells were blocked with 3% BSA in PBS-Tween 20. The samples were dispensed in duplicate into the wells. A standard curve was prepared using IFN-γ (Genzyme, Cambridge, MA) or IL-10 (BD PharMingen) for each individual plate. Cytokine production was detected by the addition of a secondary biotinylated Ab (IFN-γ clone XMG1.2, IL-10 clone SXC-1; BD PharMingen) and followed by avidin-peroxidase (Zymed Laboratories, San Francisco, CA) and 3,3′,5,5′-tetramethylbenzidine substrate (DAKO, Carpinteria, CA).

PCR analysis of mRNA

A portion of lung tissue was suspended in Ultraspec (Cinna/Biotecx, Friendswood, TX), homogenized, and frozen rapidly for storage at −70°C.

[3] Abbreviations used in this paper: CFP, culture filtrate protein.

Total cellular RNA was extracted and reverse transcribed using murine Moloney leukemia virus reverse transcriptase (Life Technologies). PCR was performed with specific primers for IL-2 or IL-10. The PCR product was Southern blotted and probed with specific labeled oligonucleotides, and the blots were developed using the ECL kit (Amersham, Arlington Heights, IL). The hypoxanthine phosphoribosyltransferase housekeeping gene was also amplified for each sample and used to confirm that equivalent amounts of readable RNA were present in all the samples. Alternatively, the detection of IL-10, TNF, or IL-12p40 mRNA was conducted using a TaqMan 7700 real-time PCR machine (Applied Biosystems, Foster City, CA). For quantification purposes, Ribosomal DNA for each sample was also assayed as an endogenous normalizer. Quantification of message was conducted using the Delta Delta Ct method.

Statistical analysis

Statistical significance was determined with the Student's t test and was found to be significant when $p < 0.05$, or highly significant when $p < 0.005$.

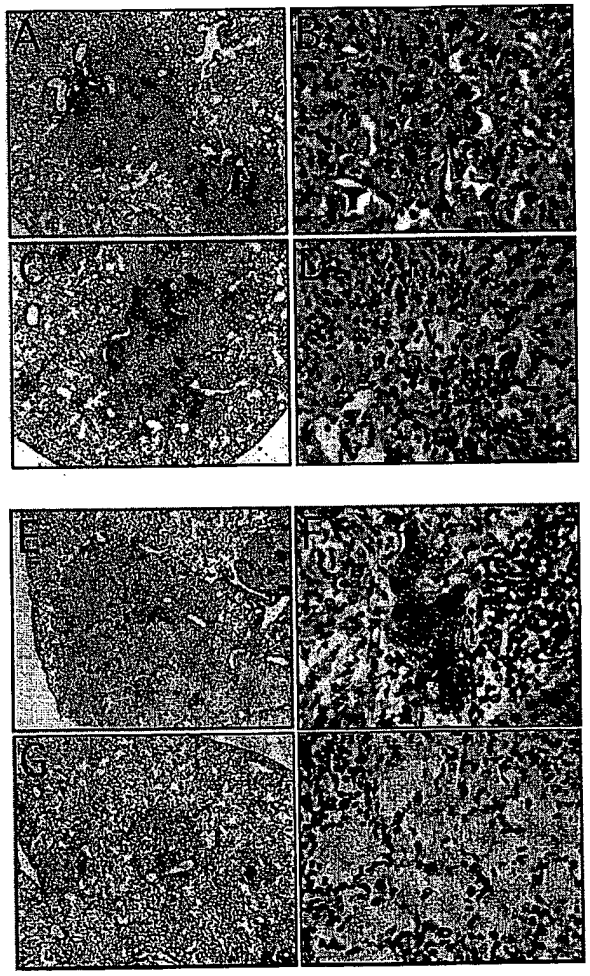


FIGURE 1. CBA/J mice infected with *M. tuberculosis* produce abundant IL-10 within the macrophage-dominated lesions of the lung. Lung tissue sections from CBA/J and C57BL/6 mice were collected 50 and 150 days postinfection with *M. tuberculosis*. Tissue was sectioned and labeled with anti-IL-10. IL-10 was found within the foamy macrophages at the center of the granuloma of CBA/J mice after 50 (*A* and *B*), and 150 (*E* and *F*) days infection. Very little IL-10 was detected within the lungs of C57BL/6 mice after 50 (*C* and *D*) and 150 (*G* and *H*) days infection. Figures are representative of three independent experiments. Magnification ×40 (*A*, *C*, *E*, *G*) and ×400 (*B*, *D*, *F*, *H*).

Results

CBA/J mice produce more IL-10 within the lesions of the lung during infection with M. tuberculosis

To determine whether the production of IL-10 could contribute to the susceptibility of CBA/J mice to reactivate *M. tuberculosis* infection, we stained sections of lung tissue from CBA/J and C57BL/6 mice for the expression of IL-10. As early as 50 days postinfection with *M. tuberculosis*, high levels of IL-10 could be detected within the lesions of CBA/J mice (Fig. 1, *A* and *B*). IL-10 was predominantly found within the macrophage populations at the center of the lesions, and could also be detected within bronchoepithelial cells lining the airways. After 150 days of infection with *M. tuberculosis*, abundant IL-10 could be detected in macrophages within the lung lesions from CBA/J mice (Fig. 1, *E* and *F*). In contrast, the lung lesions of C57BL/6 mice contained only occasional macrophages and endothelial cells that stained weakly positive for IL-10 after 50 (Fig. 1, *C* and *D*) or 150 days (Fig. 1, *G* and *H*) postinfection.

IL-10 transgenic mice are susceptible to reactivation tuberculosis

To determine whether naturally reactivation-resistant C57BL/6 could reactivate *M. tuberculosis* infection if they produced increased IL-10, we infected mice that were capable of overexpressing IL-10 (IL-10 transgenic mice) and monitored the bacterial growth over time. Whereas C57BL/6 mice were able to contain the bacterial load within the lungs for the duration of the experiment, the lung bacterial burden of IL-10 transgenic mice increased significantly during the latter part of the study to ~2 log more than the controls (Fig. 2). The increase in bacterial numbers within the lungs of IL-10 transgenic mice occurred at a similar rate to that seen in the reactivation-susceptible CBA/J mouse strain.

IL-10 transgenic mice produce more IL-10

To determine whether IL-10 was being produced in response to mycobacterial Ag, lung cells were harvested from individual mice

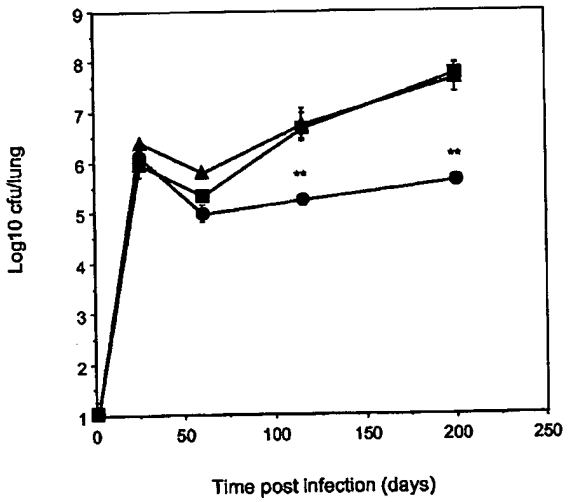


FIGURE 2. IL-10 transgenic mice are susceptible to reactivation tuberculosis. C57BL/6 (●) CBA/J (■) and IL-10 transgenic mice (▲) were infected with ~10^2 viable *M. tuberculosis* Erdman via the respiratory route and the CFU within the lung calculated at specific time points postinfection. Data represent the mean ± SEM from four mice at each individual time point and are representative of three independent experiments. Statistical significance was calculated and found to be highly significant (**, $p < 0.005$) between C57BL/6 mice and both CBA/J and IL-10 trangenic mice.

and incubated with CFPs from *M. tuberculosis*. Lung cells from IL-10 transgenic mice produced significantly more IL-10 than wild-type C57BL/6 mice both 21 and 50 days postinfection when cultured with CFP (Fig. 3). Ag-specific IL-10 production could not be detected in lung cell cultures from CBA/J mice. Comparable levels of IL-2 mRNA could be detected within the lungs of C57BL/6 and IL-10 transgenic mice during the infection, and IL-10 transgenic mice produced up to 4-fold more IL-10 mRNA within the lungs compared with wild-type mice, as determined by RT-PCR (data not shown).

CD11a and CD54 expression on CD4 T cells from the spleen and lung

We have previously demonstrated that circulating lymphocytes isolated from reactivation-prone mice failed to express the adhesion molecules CD11a and CD54 as brightly on their surface as similar cells from C57BL/6 mice (10). Indeed, IL-10 exposure has also been shown to decrease the expression of CD11a on the cell surface of lymphocytes (27). CD4 T cells isolated from the spleen of CBA/J mice expressed less CD11a on their surface (Fig. 4), as we have previously demonstrated (10). The failure to increase CD11a expression was evident up to 120 days postinfection, demonstrating that this was not a transient event in the CBA/J mouse strain. CD4 T cells from the spleens of IL-10 transgenic mice expressed a moderate expression of CD11a on their surface, equivalent to CD4 T cells from the spleens of C57BL/6 mice (Fig. 4).

Analysis of the local influence of IL-10 production on the expression of CD11a within the lungs demonstrated that CD4 T cells from both CBA/J and IL-10 transgenic mice expressed less CD11a on their surface than CD4 T cells isolated from the lungs of C57BL/6 mice (Fig. 5, *A* and *B*). In addition, lung digests revealed that CBA/J and IL-10 transgenic mice had fewer CD4 T cells within the lungs in comparison to the C57BL/6 mouse strain (Fig. 5*C*).

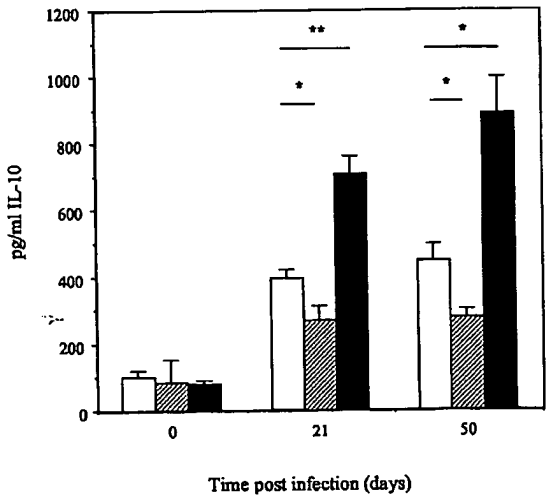
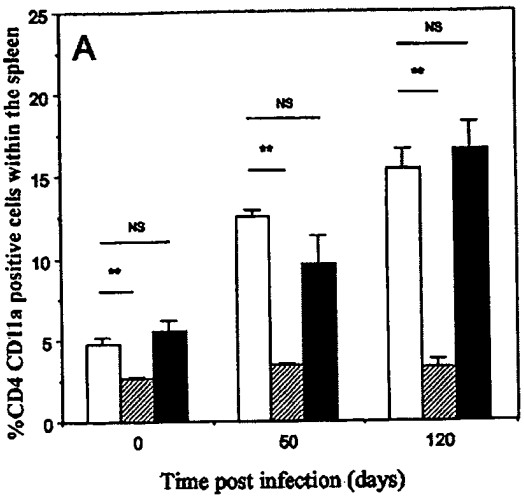


FIGURE 3. IL-10 transgenic mice produce more IL-10 in response to infection with *M. tuberculosis*. Lung cells from individual C57BL/6 (□), CBA/J (▨), or IL-10 transgenic mice (■) were cultured with CFP from *M. tuberculosis* for 5 days at 37°C, 5% CO_2. IL-10 secretion in the culture supernatants was measured by ELISA and expressed as the mean ± SEM for four individual mice at each time point. Results are representative of three independent experiments. Statistical significance was determined between C57BL/6 mice and CBA/J or IL-10 transgenic mice and found to be significant (*, $p < 0.05$) or highly significant (**, $p < 0.005$) where indicated.



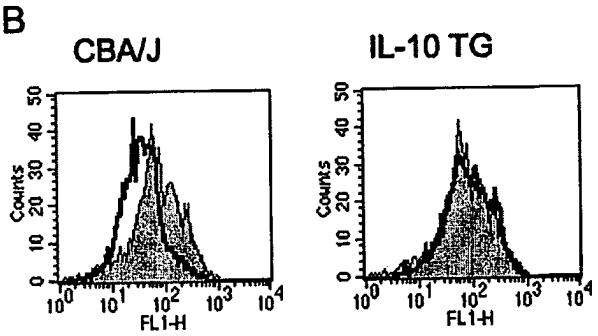


FIGURE 4. Expression of CD11a^{bright} on CD4 T cells isolated from the spleen. Spleen cells were isolated from *M. tuberculosis* infected C57BL/6 (□), CBA/J (▨), or IL-10 transgenic (■) mice throughout the course of the study and labeled with fluorescent Ab specific for CD4 and CD11a. Data are expressed as the percentage of CD4 positive cells that expressed high levels of CD11a on their surface (*A*) and represents the mean ± SEM from four mice at each individual time point. Results are representative of two independent experiments. Statistical significance was calculated and found to be highly significant (**, $p < 0.005$) between C57BL/6 mice and CBA/J mice where indicated. Representative histograms depicting expression of CD11a on CD4 T cells after 120 days infection with *M. tuberculosis*. C57BL/6 (shaded histogram), CBA/J and IL-10 transgenic (open histogram) are shown (*B*). TG, transgenic.

IL-12p40 and TNF mRNA is reduced in the lungs of IL-10 transgenic and CBA/J mice

IL-10 production can down-regulate macrophage responses, which subsequently influence the capacity of the host to generate a T cell-mediated response. To determine whether IL-10 overproduction could influence the immune response within the lungs of IL-10 transgenic or CBA/J mice, we quantified the amount of TNF and IL-12p40 mRNA during the course of infection. The overexpression of IL-10 in the IL-10 transgenic mice correlated with delayed expression of TNF mRNA in the lungs of these mice when compared with the expression in C57BL/6 mice (Fig. 6*A*). The expression of TNF mRNA was more substantially delayed in CBA/J mice, and this also correlated with the high levels of IL-10 seen in the lung lesions of these mice (see Fig. 1). The pattern of IL-12p40 expression in both the IL-10 transgenic and CBA/J mice was markedly different from that seen for the C57BL/6 mice in that

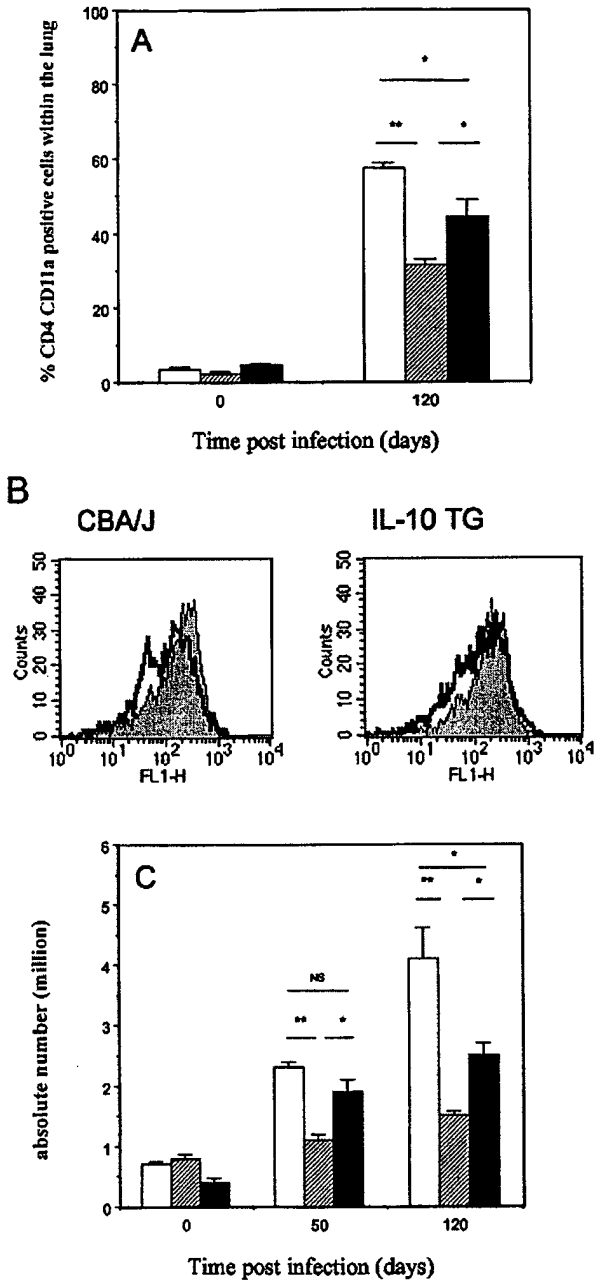


FIGURE 5. Expression of CD11a^{bright} on CD4 T cells isolated from the lung. Lung cells were isolated from *M. tuberculosis*-infected C57BL/6 (□), CBA/J (▨), or IL-10 transgenic (■) mice throughout the course of the study and labeled with fluorescent Ab specific for CD4 and CD11a. Data are expressed as the percentage of CD4 positive cells that expressed high levels of CD11a on their surface (*A*) and represents the mean ± SEM from four mice at each individual time point. Results are representative of three independent experiments. Statistical significance was calculated and found to be significant (*, $p < 0.05$), or highly significant (**, $p < 0.005$), between C57BL/6 mice and CBA/J mice, or C57BL/6 mice and IL-10 transgenic mice, where indicated. Representative histograms depicting expression of CD11a on CD4 T cells after 21 days infection with *M. tuberculosis* are shown. C57BL/6 (shaded histogram), CBA/J or IL-10 transgenic (open histogram) (*B*). Absolute number of CD4 T cells within the lungs of C57BL/6 (□), CBA/J (▨), or IL-10 trangenic (■) mice are shown (*C*).

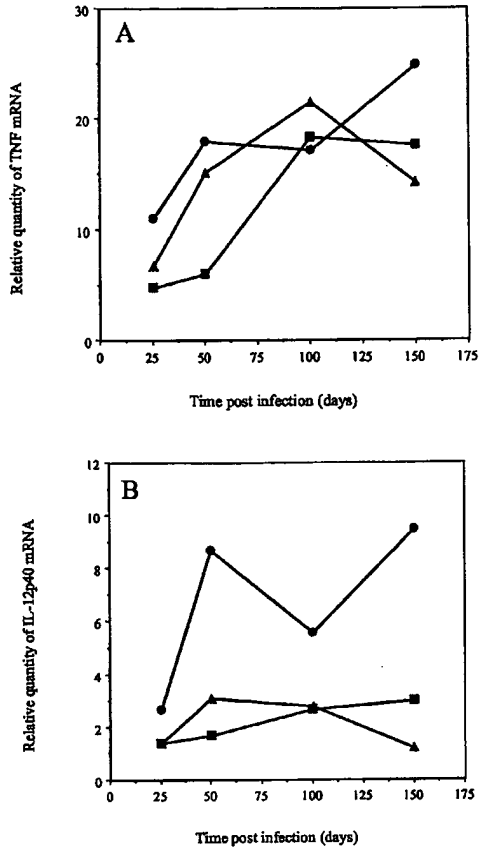


FIGURE 6. TNF and IL-12p40 mRNA production within the lung during infection. Lung tissue was obtained from C57BL/6 (●), IL-10 transgenic (▲), or CBA/J (■) mice throughout the course of infection. RNA was extracted and analyzed using primers and probes specific for TNF (*A*) or IL-12p40 (*B*). Data were normalized for the amount of RNA added to the reaction using 18S as a control.

both mice expressed low levels of IL-12p40 mRNA in the lungs throughout infection (Fig. 6*B*).

The capacity to produce IFN-γ is reduced in IL-10 transgenic mice

By its immunoregulatory action on the macrophage, IL-10 can also influence Ag-specific IFN-γ production (11, 12); therefore, we determined whether mycobacterial-specific IFN-γ production was also reduced in the lungs of IL-10 transgenic mice. Lung cells were isolated from mice that had been infected with *M. tuberculosis* and cultured with CFP Ags from *M. tuberculosis*. Ag-specific IFN-γ production was reduced in the lung cell cultures of CBA/J and IL-10 transgenic mice when compared with similar cultures from C57BL/6 mice (Fig. 7).

IL-10 transgenic mice generate macrophage-dominated lung lesions during chronic M. tuberculosis *infection*

A major characteristic of *M. tuberculosis* infection in the reactivation prone mouse strains is the predominance of macrophage type lesions within the lung. To determine whether the increased expression of IL-10 and associated decreased expression of CD11a on lymphocytes was associated with the formation of macrophage-dominated lung lesions, lung tissue was collected, sectioned, and stained with H&E. C57BL/6 mice developed characteristic lesions

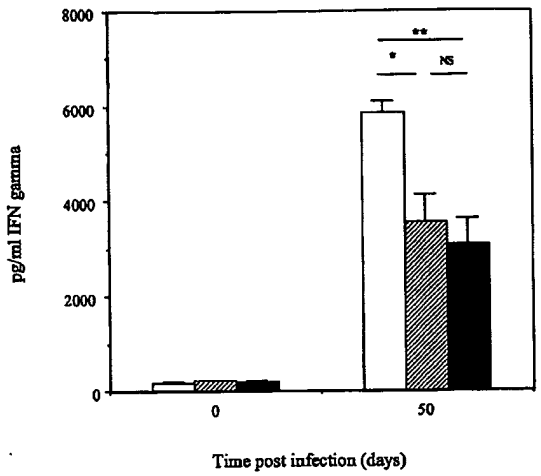


FIGURE 7. IL-10 transgenic mice produce less IFN-γ in response to infection. Lung cells from individual C57BL/6 (□), CBA/J (▨) or IL-10 transgenic mice (■) were cultured with CFP from *M. tuberculosis* for 5 days at 37°C, 5% CO_2. IFN-γ secretion in the culture supernatants was measured by ELISA and expressed as the mean ± SEM for four individual mice at each time point. Results are representative of three independent experiments. Statistical significance was calculated and found to be significant (*, $p < 0.05$), or highly significant (**, $p < 0.005$), between C57BL/6 mice and CBA/J mice, or C57BL/6 mice and IL-10 transgenic mice, where indicated.

(25) consisting of epithelioid macrophages associated with prominent intralesional scattered or aggregated areas of lymphocytes that persisted until at least 200 days (Table I and Fig. 8, *A* and *B*). Initially, the lesions within the lungs of IL-10 transgenic mice resembled those seen in the C57BL/6 strain with prominent lymphocytic perivascular/peribronchiolar accumulations and abundant macrophage clusters (Fig. 8*C*, day 50). However, of particular interest was the observation that after 200 days of infection with *M. tuberculosis*, only a few loose lymphocyte aggregates were observed. The lesions had progressed to consist predominantly of foamy macrophages (Fig. 8*D*, day 200) with cellular necrosis, and cholesterol deposition similar to that observed in the lungs of reactivation-prone mouse strains.

Discussion

In these studies, we demonstrate that IL-10 could be detected within the lesions of CBA/J mice as early as 50 days postinfection with *M. tuberculosis*, suggesting that IL-10 may influence the ensuing immune response and subsequent susceptibility to reactivation disease in this mouse strain. To fully determine whether IL-10 had any influence on the course of chronic tuberculosis, we infected C57BL/6 transgenic mice that express IL-10 under control of the IL-2 promoter (26) with *M. tuberculosis*. Infection resulted in the increased production of IL-10 within the lungs of IL-10 transgenic mice. The most significant finding was that although IL-10 transgenic mice could initially control an infection with *M. tuberculosis*, as the period of infection progressed into the chronic/latent phase they began to show signs of reactivation disease. Therefore, these findings demonstrate that the addition of a single cytokine, IL-10, resulted in a reactivation-susceptible phenotype in a naturally reactivation-resistant C57BL/6 mouse strain. Our results also confirm findings by Murray et al. (28) in which the overexpression of IL-10 led to increased growth of *M. bovis* bacillus Calmette-Guérin within the spleen and liver of mice. These data support the hypothesis that the increased production of IL-10 is a potential immune correlate of susceptibility to reactivation tuberculosis, and may be the underlying trigger for this event.

In a mouse model of progressive primary tuberculosis, IL-10 was not detected until at least 6 mo (180 days) following an infection with *M. tuberculosis* (29), and we demonstrate in this study that the C57BL/6 mouse strain produced very little IL-10 during chronic *M. tuberculosis* infection. These results suggest that the production of IL-10 is not associated with the initial control of infection, but that this cytokine may be more important during the chronic or latent phase of tuberculosis in this model. Therefore, this may explain why only a moderate and transient enhanced early resistance can be found when C57BL/6 IL-10 gene-disrupted mice are infected with mycobacteria (20, 21, 23, 30). This increased resistance to infection in the absence of IL-10 is documented to be associated with an increased expression of inflammatory mediators such as TNF (21), inducible NO synthase (21), and IL-12 (23).

Table I. *Analysis of microscopic lung lesions from* M. tuberculosis*-infected mice*

Time Postinfection (days)	Mouse Strain	
	C57BL/6	IL-10 Transgenic
25	Mild Perivascular/brochiolar lymphocytes. Individual foci of epithelioid macrophages.	Mild Lymphocytic lesions with fewer epithelioid macrophages. Increased number of neutrophils.
50	Moderate Prominent intralesion scattered or aggregates of lymphocytes associated with epithelioid macrophages.	Moderate Perivascular/bronchiolar lymphocytes associated with abundant macrophages. Moderate macrophage vacuolation.
150	Moderate Markedly vacuolated epithelioid macrophages. Occasional giant cells. More prominent necrosis and slight increase in neutrophils. Early extracellular cholesterol cleft formation.	Marked Extensive coalescing lesions consisting of markedly vacuolated macrophages. Lymphocytes associated with small foci of necrosis. No significant cholesterol formation.
200	Moderate Lymphocytes dominate and are either loosely organized within the lesions, or in dense aggregates. Vacuolated macrophages.	Marked >80% lung involvement. Loosely dispersed lymphocytes with degenerating macrophages. Prominent cholesterol clefts. Mixed inflammatory cells in the airways.

[a] Lung tissue was harvested from *M. tuberculosis*-infected mice at the designated time points. Tissue was sectioned, stained with H&E, and interpreted by a veterinary pathologist without prior knowledge of the groups. Tissue sections were graded according to gross evaluation of lesion size, number, and distribution. Data are representative of two independent experiments.

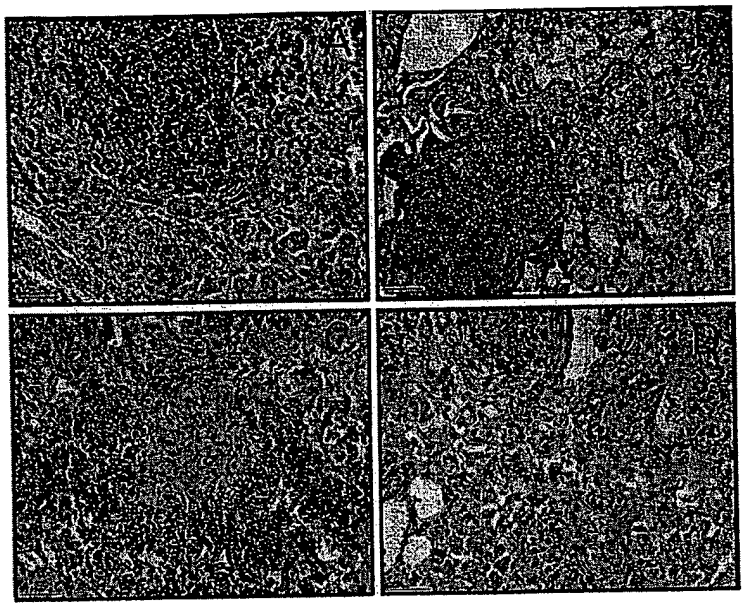


FIGURE 8. IL-10 transgenic mice generate macrophage-dominated lesions during chronic *M. tuberculosis* infection. Formalin-fixed tissue was prepared and sectioned for light microscopy. The tissue was stained with H&E. C57BL/6 mice developed characteristic lesions consisting of epithelioid macrophages associated with prominent intralesional scattered lymphocytes (*A*; day 50), that persisted throughout the infection (*C*; day 200). The lungs of IL-10 transgenic mice showed perivascular/peribronchiolar lymphocytes associated with abundant macrophage clusters (*B*; day 50) which developed into lesions consisting predominantly of foamy macrophages, with cholesterol deposition and necrosis (*D*; day 200). Magnification ×200, bar = 100 μm. Figures are representative of two independent experiments.

Although IL-10 appears to reduce the production of these and other inflammatory molecules (16, 31), the transient and minimal reduction in bacterial numbers early in the course of infection of IL-10-deficient mice suggests that this molecules does not act to limit early protective responses in vivo.

Although early protective mechanisms appear unaffected by IL-10, we clearly show in this study that the expression of IL-10 during chronic infection is detrimental to both the protective antibacterial response and to the development of stable mononuclear granulomas. The overexpression of IL-10 in the IL-10 transgenic mice clearly correlates with the reduced expression of the cytokines TNF and IL-12p40, which are not only antibacterial, but also important in the generation of stable granulomas (32–34). That this reduced cytokine expression is also seen in the CBA/J mice, which also expressed high levels of IL-10 in the lung (albeit from macrophages rather than T cells), suggests that IL-10 is an active down-regulator of protective responses in the chronically infected murine lung. Why the early protective response is unaffected by IL-10 expression when the response within the chronically infected lung is altered poses an interesting question for further study. It is likely that the potent TH1-inducing nature of *M. tuberculosis* (33, 35) overrides any early inhibitory IL-10 effects, but that as disease develops, the levels of IL-10 increase (artificially in the IL-10 transgenic and naturally in the CBA/J mice) and the cytokine is able to limit the expression of protection.

Enhanced resistance to infection with other pathogens has also been demonstrated in IL-10 gene-disrupted mice (36, 37). However, it is also apparent that despite reducing bacterial numbers, the failure to down-regulate an acute immune response can also be fatal to the host due to the generation of an overwhelming inflammatory response (38–40). That IL-10 does not appear to be essential for the control of an infection with *M. tuberculosis*, or that its absence does not result in progressive inflammatory responses within the lung suggests that the down-regulation of the immune response to *M. tuberculosis* could be mediated by an independent mechanism. Alternatively, we demonstrate in this study that the overexpression of IL-10 results in the exacerbation of disease; therefore, it seems possible that a continued immune response during chronic *M. tuberculosis* infection may in fact be critical for the containment of a long-term infection. Studies that have sought to determine the immune parameters that are required for the maintenance of a chronic infection with *M. tuberculosis* have identified numerous molecules that include T cell subsets (41, 42), TNF (43, 44), and NO (45). It is also of interest to note that these same molecules are required for the initial control of infection (46–48), adding further support to the hypothesis that a continued immune response is necessary to prevent the reactivation of infection in the mouse model.

As we have demonstrated, the overexpression of IL-10 in our model was associated with a reduced capacity of lung cells to produce IFN-γ in vitro in response to CFP Ags from *M. tuberculosis*, thus demonstrating that the Ag-specific response could be compromised. The presence of IL-10 within the lungs of infected mice also inhibited the further recruitment of lymphocytes into this organ, demonstrated by the finding that in comparison to wild-type mice, overexpression of IL-10 resulted in fewer CD4 T cells within the lungs during the infection. The production of IFN-γ per cell was not dramatically altered (C57BL/6 1.4×10^{-3}; CBA/J 1.6×10^{-3}; IL-10 trangenic 0.98×10^{-3} pg/ml); however, the significantly reduced number of T cells within the lungs of IL-10 transgenic and CBA/J mice would also result in a net reduction of IFN-γ within the lungs.

Perhaps of more importance was the finding that those CD4 T cells present within the lungs of IL-10 transgenic mice also failed to up-regulate the expression of CD11a on their surface. IL-10 has been shown to influence the expression of CD11a on the surface of T cells (27), and may have a local influence on lymphocytes as they enter the lungs, down-regulating the expression of CD11a and influencing the capacity of lymphocytes to migrate within the lesions. This failure to up-regulate the adhesion molecule CD11a resembles the phenotype of two mouse strains, CBA/J and DBA/2, that we have previously described as reactivation-susceptible (10). The reduced expression of CD11a on T cells from CBA/J mice is a systemic effect as shown in this study and previously (10). That the IL-10 trangenic mice did not exhibit reduced CD11a expression in the spleen (where the level of IL-2 induction, and thus IL-10, is reduced due to low levels of infection and thus, immune stimulation) supports the hypothesis that it is IL-10 expressed at the site of infection that is limiting the expression of CD11a on the T cells within the lungs of the IL-10 transgenic mice. Therefore,

these studies identify the overproduction of IL-10 as a potential correlate of reactivation tuberculosis in the mouse model that results in the characteristic phenotype within the lungs that is seen in the naturally reactivation-susceptible mouse strains.

The loss of lymphocyte foci within the lungs of IL-10 transgenic mice appears to have a significant impact on the integrity of the lung lesions as the course of infection ensued. The absence of localized T cells and the loss of Ag-specific IFN-γ production appeared to be directly associated with the gradual increase in bacterial load within the lungs. As this occurred, lung lesions in the IL-10 transgenic mice were dominated by macrophages and with progressive degeneration, characterized by necrosis and neutrophil influx. As such, these lesions closely resembled the macrophage-dominated lesions that we have previously described in two reactivation-prone mouse strains (10). The overexpression of IL-10 within lesions of the lung, whether via transgene expression or as a result of IL-10-producing macrophages within the lesions, thus clearly predisposes these mice to enter a stage of reactivation disease.

The identification of immune correlates of protection/susceptibility to tuberculosis in the mouse model takes on particular importance if these findings can be extrapolated to man. The identification of individuals that have the potential to reactivate a latent *M. tuberculosis* infection, perhaps by a significant increase in their capacity to produce IL-10, may help identify individuals who will go on to develop reactivation disease later in life and hence be a source of infection. Therapeutic treatment of these individuals with drugs or perhaps with anti-IL-10R Ab (49) could potentially prevent reactivation tuberculosis and thereby reduce the index cases for infectious tuberculosis within the community. In support of this concept, elevated levels of serum IL-10 have been reported in individuals with active tuberculosis (19) and in the pleural fluid of tuberculosis pleurisy patients (18). In addition, elevated IL-10 levels appear to be greater in individuals that are coinfected with *M. tuberculosis* and HIV (50–52), which reflects the ability of both *M. tuberculosis* and HIV to stimulate IL-10 production during infection (53). Therefore, observations that the production of IL-10 is associated with the development of active (or reactivation) disease in man are in keeping with the findings of the present study, which demonstrate that increased susceptibility to reactivation tuberculosis in the mouse model is strongly influenced by the expression of IL-10 during the chronic or latent phase of the infection.

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Links

Virulence of Mycobacterium tuberculosis affects interleukin-8, monocyte chemoattractant protein-1 and interleukin-10 production by human mononuclear phagocytes.

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Microbial virulence and cytokine-mediated immune responses to Mycobacterium tuberculosis infection are important determinants of the pathogenesis of human tuberculosis. To determine the interrelationship between mycobacterial virulence and cytokine induction, human monocytes and monocyte-derived macrophages were infected with attenuated (H37Ra) and virulent (H37Rv and CH306) strains of M. tuberculosis and the amount of proinflammatory [interleukin (IL)-8 and monocyte chemoattractant protein (MCP)- 1] and inhibitory (IL- 10) cytokines was measured in the culture supernatants by enzyme-linked immunosorbent assay (ELISA). Infection with live bacilli induced de novo synthesis of IL-8, MCP-1 and IL-10, since cytokine release was abolished when cells were preincubated with the protein synthesis inhibitor cycloheximide. A differential production of antiinflammatory and inhibitory cytokines was observed. The amount of IL-8 and MCP-1 release was inversely related to strain virulence, the attenuated H37Ra strain being more prone than virulent strains to induce secretion of chemokines. In contrast, virulent strains induced greater amounts of the inhibitory cytokine IL-10. Efficient upregulation of IL-10 synthesis, but not of chemokines, required infection of cells with live bacilli, since heat killing of organisms or challenge with soluble mycobacterial products completely abrogated the effect. Moreover, cells infected with virulent strains produced IL-10 even at a very low bacillus-to-cell ratio and secreted IL-10 continuously during the 96 h that followed infection. The results suggest that the degree of virulence affects host cell responses to M. tuberculosis infection. Continued production of IL-10 may be one of the means by which M. tuberculosis downregulates acute local inflammatory reactions, favoring the development of tuberculosis.

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